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FINE NEEDLE BIOPSY DIAGNOSIS IN NODULAR PULMONARY AMYLOIDOSIS

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Primary isolated nodular pulmonary amyloidosis is a rare condition of unknown aetiology The disease runs a benign course but offers a diagnostic problem since it often simulates neoplasm A case is reported in which the initial diagnosis of a suspected squamous cell carcinoma was made on the basis of material obtained by transthoracic needle biopsy Lobectomy was performed and the histopathological and histochemical examinations gave the diagnosis of amyloidosis Re examination of the aspiration biopsy material demonstrated the possibilities of making a cytological diagnosis of amyloidosis

Amyloid deposits in the upper respiratory tract are not uncommon and according to reports have been observed in more than 100 patients Primary isolated nodular amyloidosis of the lung parenchyma however, is rarely encountered *Firestone & Joison* (1966) have found 28 cases in the literature, including their own Most of these were diagnosed at autopsy The patients are usually over fifty years old and both sexes are equally represented Among the seven cases diagnosed during life four have been asymptomatic They were detected at routine roentgenological chest examination The final diagnosis of amyloid tumour was made by histological examination of tissue obtained by bronchoscopic biopsy or thoracotomy

The diagnosis is impossible to make solely by radiological examination The usual preliminary diagnoses are Benign or malignant tumours or pulmonary tuberculosis Transthoracic needle biopsy during TV fluoroscopy (*Dahlgren & Nordenstrom* 1966) offers a relatively simple method for cytological diagnosis of pulmonary lesions This diagnostic possibility has not previously been discussed in the literature in connection with pulmonary amyloidosis

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CASE REPORT

Clinical Findings

The patient was a 66 year-old woman asymptomatic previously in good health At routine chest examination in December 1966 a well defined lesion was found in the left lower lobe

Physical examination showed a normally built woman with no signs of heart decompensation blood pressure 150/90 pulse 75 A diastolic murmur was heard Auscultation of the lungs was normal. Laboratory tests Hb 14 5 per cent white blood cell count 5 200 with a normal differential blood count protein electrophoresis normal sedimentation rate 20 mm Pulmonary liver and renal function tests were normal

Radiological Findings

Chest examination in January 1967 showed a small round infiltrate in the basal lateral segment of the left lower lobe with a diameter of about 2½ cm (Fig 1) The lesion did not contain any calcification and did not change in volume with the Valsalva manoeuvre No surrounding atelectasis was seen and no hilar adenopathy was identified No changes were present in the parenchyma of the right lung The total heart volume was nor

Fig 1 Chest examinations demonstrate a rounded infiltration in the left lower lobe adjacent to the diaphragm The lesion has a diameter of about 2 5 cms Lateral projection

mal but a ght left ventricular dilation and widening of the ascending aorta was present

Earlier mass chest examination in December 1964 revealed no changes in the lungs In December 1966 an infiltration of the same size and configuration as in January 1967 was present in the left lower lobe The infiltrate was suspected to be a peripheral malignant tumour A transthoracic needle biopsy was performed under TV fluoroscopy to obtain a definite diagnosis A mediastinal jugular cannulation during TV fluoroscopy according to *Nordenstrom* (1967) was performed in order to demonstrate possible mediastinal lymph node metastases

Pathological Findings

Cytological examination of material from the transthoracic needle biopsy demonstrated atypical epithelial cells which gave a suspicion of squamous cell carcinoma The mediastinal biopsy was entirely negative Thoracotomy was performed The left lower lobe was removed with some lymph nodes from the hilar region The lobe measured about 12 × 10 × 8 cm It was covered by normal pleura except for some scarring on the diaphragmatic surface Basally and sub-pleurally there was a well demarcated firm and grayish white nodule The lesion measured 2 5 cm in diameter In the parenchyma surrounding the lesion two more nodules were found These had a diameter of about 0 5 cm The rest of the lung parenchyma was normal The lymph nodes in the hilar region were anthracotic but otherwise normal

Microscopically the three nodules contained amorphous eosinophilic material Staining with Congo red Periodic Acid Schiff and methyl violet



Fig 2 Peripheral portion of the amyloid deposits The lesion is well demarcated but not encapsulated In the surrounding lung parenchyma there is a moderate lymphocytic infiltration × 80

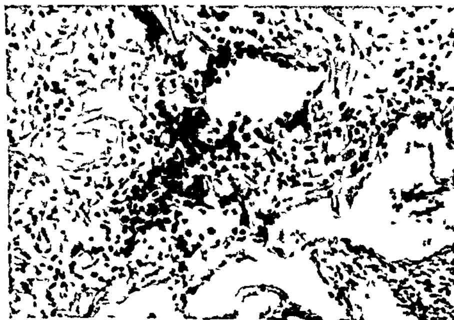


Fig 3 Peripheral portion of the amyloid deposits Note the proliferating and atypical epithelium in some small bronchi × 200



Fig 4 Atypical squamous epithelial cells found in the aspiration biopsy material × 500

gave reactions clearly positive for amyloid Small amounts of fibrous tissue and some blood vessels were also present The amyloid deposite were well demarcated but not encapsulated The surrounding lung parenchyma was moderately infiltrated with lymphocytes and granulocytes but there were only a few plasma cells (Fig 2) Foreign body giant cells could be seen There were no necrotic areas or calcification The alveolar lining cells in the vicinity of the lesion were irregular and hyperplastic and the lining in some small bronchi contained metaplastic squamous epithelium (Fig 3) These cells were principally of the same type as those found at the previously performed needle biopsy (Fig 4) and the reason why some atypical cells were suspected of being malignant Except for the local deposits described no amyloid substance was observed in the lung parenchyma the walls of the bronchi or the vessels There was no evidence of systemic amyloidosis

The aspiration biopsy material was re-examined Small amounts of amorphous weakly eosinophilic material could be demonstrated in all of the four slides This material was primarily assessed as necrotic like material The appearance however was not typical for necrotic material seen in tuberculosis or in tumour-cases Two of the primary haemotoxylin-eosin stained slides were destained and restained with Congo red and Periodic Acid Schiff The amorphous material gave positive reactions for amyloid (Fig 5)

The atypical epithelial cells seen at the primary examination represent quite probably chronically *irritated cells from the bronchi in the vicinity of* the amyloid lesion

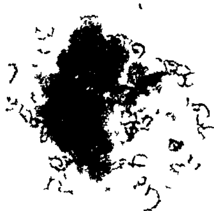


Fig 5 Fragments of amorphous material found in the aspiration biopsy slides This material gave amyloid positive reactions with Congo red and Periodic Acid Schiff × 500

DISCUSSION

The main differential diagnosis in cases of isolated nodular pulmonary amyloidosis is malignant tumour (*Cotten & Jackson* 1964, *Haynes et al* 1948) As in cancer of the lung the patient is often symptomfree and the reason for further examination is a positive routine x ray Symptoms when present often indicate a bronchiostenosis like cough haemoptysis recurrent pneumonias and dyspnoea (*Condon et al* 1964, *Crater* 1965)

Radiological findings are not typical In most cases a rounded relatively well delineated infiltration is present either in the central or in the peripheral part of the lung In that respect the lesions has the same appearance as primary or metastatic carcinoma of the lung or tuberculoma

In the present case the localization and the radiological appearance in a patient in the age group of 60 gave the suspicion of malignant tumour Benign tumour or tuberculosis has also to be considered as a possible diagnosis Tuberculine test and the guinea pig inoculation with gastric washing however did not give any evidence of tuberculosis

The next procedure to obtain a diagnosis in such cases is a transthoracic needle biopsy (*Dahlgren & Nordenstrom* 1966) The experience from the above case demonstrates *that it is possible to get* a diagnosis of amyloidosis with the aid of aspiration biopsy Special

uning, however, must be performed It is importance therefore that the cytologist is of the diagnosis and that cases with ious non characteristic necrotic like I request special stains

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EFFECT OF ANTI-ANGIOTENSIN II ON BLOOD PRESSURE AND SENSITIVITY TO ANGIOTENSIN AND RENIN

Studies on Normal Nephrectomized Ureterligated Continuously Angiotensin Infused, and Renal Hypertensive Rats

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1 Intravenous injection of anti angiotensin II causes a transient fall in the mean blood pressure of *anaesthetized normal rats* showing that the angiotensin content in the blood plays a role for the normal blood pressure level The subsequent return of the blood pressure to its initial level is probably caused by the cardio-accelerator and pressor reflexes The function of the baroreceptors is also thought to be the cause why a depressor effect of anti angiotensin is lacking in *conscious normal* rats Lack of effect on *anaesthetized nephrectomized* rats conforms to the lack of angiotensin in their blood The somewhat increased depressor response to anti angiotensin in rats with *acute severe constriction* of the renal arteries and *the still more pronounced response in many* (but not all) *renal hypertensive rats conforms to* increased plasma angiotensin in these rats The somewhat decreased depressor response of ureterligated rats corresponds to their decreased sensitivity to angiotensin and renin 2 Anti angiotensin causes a *decreased response to injected angiotensin* both in conscious normal and anaesthetized normal nephrectomized ureterligated and renal hypertensive rats the sensitivity in several cases being reduced to less than 1 per cent If angiotensin is *continuously infused* in doses of 9 ng/min anti angiotensin will cause a fall of the blood pressure which in one half of the cases reaches the initial level in the other half stops at a level above the initial even when a second dose of anti angiotensin is injected When anti angiotensin is given between two periods of continuous infusion of angiotensin the second rise in blood pressure will be smaller being up to 50 per cent of the first 3 Anti angiotensin causes a *decreased response to renin* but the above mentioned groups of rats do not react in the same way In anaesthetized *normal* rats the sensitivity is reduced to about 5 to 10 per cent *Nephrectomized* and *ureterligated* rats are still able to react with a pressor response to doses of renin which are without effect on the blood pressure of anti angiotensin pretreated normal rats The maximum response of rats belonging in these two groups is however only up to 50 per cent of that found in non pretreated nephrectomized or ureterligated rats If given shortly after renin anti angiotensin injection will cause a rapid fall in the blood pressure to the initial level in normal and ureterligated rats while there is only a partial return to a level markedly above the initial in nephrectomized rats

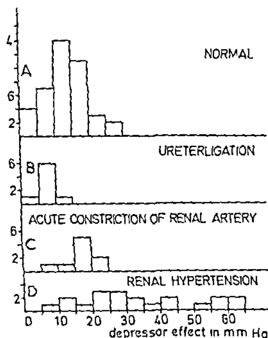


Fig 1 Abscissa The maximal depressor effect in mm Hg after injection of about 100 µg (50–500 µl) anti angiotensin in anaesth tized A) normal rats B) ureterligated rats C) rats with acute severe constriction of one or both renal arteries and D) renal hyperten sive rats Ordinate number of rats

Based on the fact that the blood pressure is unchanged by nephrectomy it has been believed that the renin system has no share in the normal blood pressure level Similarly the highly varying and in many cases normal plasma angiotensin found in renal hypertensive patients and animals have shown that the high blood pressure is not simply due to the pressor effect of an in creased amount of angiotensin in the blood

Recent studies using anti angiotensin II showed that the blood pressure of renal hy pertensive rats was uninfluenced by intra venous injection of anti angiotensin (*Hed wall* 1967 and 1968) Contrary to this it was found that an intravenous injection of anti angiotensin in anaesthetized normal rats most often resulted in a transient lowering of the blood pressure while no such effect was found in nephrectomized rats (*Bing & Poulsen 1968*) It was further shown that anti angiotensin made normal rats unrespon sive to both angiotensin and renin while nephrectomized rats were made unrespon sive to angiotensin but still responded to renin with the typical prolonged response The maximum height of this response was lower, however than in untreated nephrec tomized rats

The present study gives a more detailed report of our previously published ex periments which have been extended in cluding studies of the effect of anti angioten sin on conscious normal rats and on an aesthetized ureterligated and renal hyperten sive rats as well as on normal rats con tinuously infused with angiotensin

MATERIAL AND METHODS

Female albino rats (abou 200 g) were anaesthe tized with 20 mg of amytal and some of them pre treated with 0 005 mg of ergotamine tartrate Some further rats which were studied in the conscious state had polyethylene catheters inserted in the femoral artery and jugular vein during a short light ether anaesthesia about one hour or more before the experiment was performed The venous ca theter was placed subcutaneously around the neck and then through the skin using the method of *Bojesen* (1966) which allows the animal to move freely The blood pressure was measured with a mercury manometer in the narcotized rats and with a Tybjærg Hansen capacitance manometer in the conscious rats and in the anaesthetized rats used as their controls Nephrectomy and ureterligation were performed 21–24 hours before the experiment Renal hypertension was produced by the method of *Wilson & Byrom* (1939) using a silver ribbon clip with a 0 2 mm broad opening to constrict the left renal artery In these rats the postoperative increase in blood pressure was controlled by the tail plethysmograph method the pressure being taken at frequent intervals during recovery from a light ether anaesthesia More narrow clips with an opening of 0 15 mm were used in some experi ments in which the effect of anti angiotensin was studied 20 to 48 hours after the application

The *angiotensin* preparation was the angioten sin A 65 standard and the *hog renin preparation* was the standard of Dr *Haas* both obtainable from the WHO Lab for Biol Standard Mill Hill London The *vasopressin* preparation was the ly

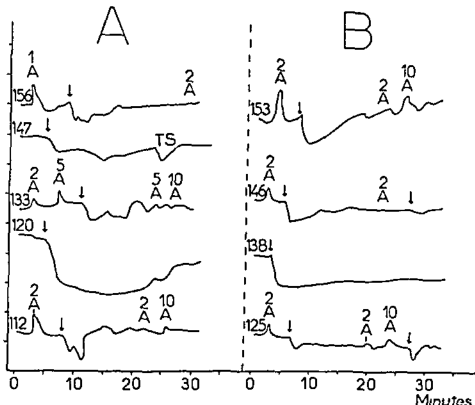


Fig 2 Effects of intravenous injection of 50 µl of anti angiotensin on blood pressure and sensitivity to angiotensin in A) normal rats (left) and B) rats with acute severe constriction of one or both renal arteries (right) Values given in front of each curve indicate the initial mean blood pressure The marks on ordinate indicate intervals of 20 mm Hg Injections of 50 µl of anti angiotensin during about 10 sec ed by an arrow Angiotensin is marked by an A the dose in ng being given above the A TS s tracheal secretion partly occluding the tracheal cannula

sine 8 vasopressin from Sandoz In most of the experiments an immune plasma from a rabbit (K 765 A) was used as *anti angiotensin II* preparation 1 ml of which neutralized c 5–10 µg angiotensin II the association constant being 5×10^{9} 1/M (*Poulsen* 1969) In some further experiments antisera from two other rabbits (K 64 E and K 072 E) were used the neutralization capacity being about 15 and 2 µg and the association constant being 2×10^{10} and 3×10^{10} 1/M respectively

For the production of anti angiotensin rabbits were immunized with Asp $(NH_2)^1$ Val^5 angiotensin II Ciba (pure substance) coupled to porcine gamma globulin (Nutritional Bioch Co) as described by *Goodfriend et al* (1964) 3 mg of this conjugate dissolved in 500 µl of saline was suspended in 500 µl of complete Freund adjuvant and given in multiple small subcutaneous injections to rabbits once a month for three months and then at intervals of 2 to 4 months the total time of immunization being 6 to 10 months

RESULTS

1 *Anaesthetized Normal Rats*

The anti angiotensin II containing plasma K 765 A was injected intraveously in doses from 20 to 500 µl into 43 rats the injections in most cases being given during about 10 seconds The injections of 50 to 500 µl resulted in a *depressor effect* the *degree* of which varied from 0 to 30 mm with a mean of about 13 mm (Fig 1) Using these doses there was no relation between dose and depressor effect, but doses of only 20 µl gave less depressor effect which could be increased by a second dose of 20 µl The *duration* of the depressor effect varied much from a few minutes to more than an hour but apart

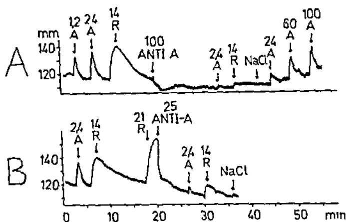


Fig 3 A In a *normal* rat injection of 100 µl of anti angiotensin (ANTI A) resulted in 1) a depression of the blood pressure and 2) an about 50 times decreased sensitivity to angiotensin (A) and renin (R) B In a *normal* rat a dose as small as 25 µl of anti angiotensin given at the time when the renin pressor curve had reached its maximum value 1) changed the normal form of the renin curve by a steep fall to a level below the initial level and 2) markedly diminished the response to both angiotensin (A) and renin (R) The angiotensin renin and anti angiotensin values in ng micro Godblatt Units and µl respectively are given above the marks

from cases in which the depressor effect was less than 5 mm and only lasting for a few minutes there was no relation between the degree and the lenght of the effect as seen in Fig 2 A The blood pressure of the rats at the time of injection of anti angiotensin varied from 85 to 155 mm but these variations were without influence on the degree

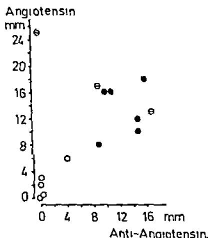


Fig 4 The pressure response in mm Hg to 2 4 ng of angiotensin (ordinate) plotted against the subsequent response to 100 µl of anti angiotensin (abscissa) in anaesthetized normal rats (●) conscious normal rats (○) and ergotamine tartrate pretreated conscious rats (⊖) With one exception depressor response to anti angiotensin follows sensitivity to angiotensin

and duration of the depressor effect (Fig 2 A) which was also uninfluenced by pretreatment of the rats with ergotamine tartrate

Similar results were obtained with the two other immune plasma (K 64 E and K 0 72 E), using K 0 72 E however required about three times as much plasma in order to obtain the maximum response

Injection of 50 to 500 µl of anti angiotensin (K 765 A) resulted in a subtotal or more often total *unresponsiveness* to 2 4 ng *angiotensin* and to 1 4 × 10 GU *renin*, which before the anti angioten in injection gave pres or effects of 10 to 25 mm Hg (Fig 3 A) When tested with 10 to 100 times the primary dose of angiotensin the anti angiotensin treated rats were either unresponsive or reacted with a response smaller than that evoked by 2 4 ng before the treatment (Fig 3 A) While the sensitivity to angiotensin in several cases was reduced to less than 1 per cent the sensitivity to renin only decreased to 5 or more often 10 per cent even in cases when threefold the usual dose of anti angiotensin was given

If the anti angiotensin was given at the time when renin pressor effect had reached its maximum the blood pressure would return to and further fall below the starting level the steepness of the fall differing markedly from the normal slower return of the renin response seen in Fig 3 B This fall was

obtained with as small a dose as 25 μl anti angiotensin In a few cases the anti angiotensin treated animals reacted to angiotensin and (or) renin injection with a depressor response The anti angiotensin injection was without effect on the response to doses of vasopressin and noradrenaline which were equipressor to 2 4 ng of angiotensin (Fig 1 A in the previous paper (1968)) Most experiments were stopped while the rats were still unresponsive to the primary doses, in nine cases 15 minutes in seven 30 minutes in seven 1 hour and in two cases two and three hours after the injection of anti angiotensin

2 *Conscious Normal Rats*

Contrary to the finding in anaesthetized normal rats the blood pressure of five conscious rats was either uninfluenced (4 cases) or only slightly depressed (1 case) after injection of 100 μl of anti angiotensin (K 765 A) One of three conscious rats which were pretreated with *ergotamin tartrate* did not respond to anti angiotensin while the other two responded with quite the same depressor effect as six anaesthetized which were studied on the same days the same *manometer* and the same dose anti angiotensin as the conscious rats se results are shown in Fig 4 which also gives the values of the pressor effects of 2 4 ng of angiotensin in the same rats obtained *before* the injection of anti angiotensin

3 *Nephrectomized Rats*

50 to 300 μl of anti angiotensin (K 765 A) did not influence the blood pressure in 12 anaesthetized nephrectomized rats In these animals the anti angiotensin produced the same unresponsiveness to angiotensin as that found in normal rats but contrary to these the nephrectomized still responded to renin The response had the sam prolonged form but a slower rise and a maximum increase of only about 50 per cent of that found in *non* anti angiotensin pretreated nephrectomized rats (Fig 5 C) The nephrectomized rats also *differed from the normal rats in the* results obtained when the renin response had reached its maximum While anti angiotensin injection would result in a quick return to the initial blood pressure level in normal animals (Fig 3 B) *even much higher doses* would only result in a smaller fall to a still significantly increased level in nephrectomized rats (Fig 5 A B and D) While 14 out of 15 nephrectomized rats reacted in this way a *single nephrectomized rat* (*Fig 5 C*) reacted differently, the blood pressure returning stepwise to the starting level after two injections of anti angiotensin

4 *Ureterligated Rats*

In 7 anaesthetized ureterligated rats injection of 100 μl of anti angiotensin (K 765 A) resulted in a mean blood pressure fall of 7 *mm (from 2 to 10 mm)* the duration of the depression in most cases being 30–45 minutes The maximum depressor effect is thus a little lower than that found in normal rats (Fig 1 B and 1 A respectively)

As previously shown (1970) *ureterligated* rats react to injection of angiotensin and renin with a pressor response lower than that of normal rats the response to renin being of a duration prolonged similarly as in nephrectomized rats In 13 ureterligated rats the injection of anti angiotensin resulted in a decrease of the sensitivity *to angiotensin* equal to that in anti angiotensin treated normal rats But contrary to findings in normal rats, and similar to the finding in anti angiotensin pretreated nephrectomized rats anti angiotensin was *unable to inhibit completely* the response to renin which was about half of that found in non anti angiotensin pretreated ureterligated rats (Fig 6 A and B) the mean values being 7 mm (from 3 to 10) and 18 *mm* (from 15 to 30) respectively While the ureterligated rats resembled the nephrectomized ones both *in the prolonged* pressor response to renin and in the ability to react to renin after injection of anti angiotensin, they differed from the nephrectomized in reacting with a return of the blood pres

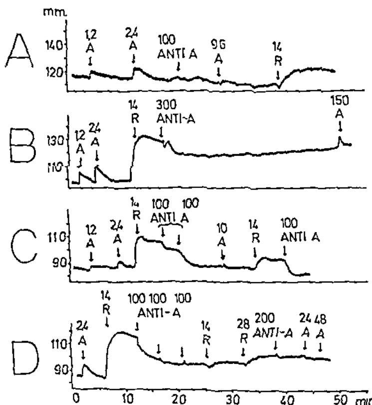


Fig 5 A In a *nephrectomized* rat 100 µl of anti angiotensin 1) does not produce any depressor effect 2) causes a pronounced decrease in sensitivity to angiotensin The animal is however still able to react to renin but the pressor re ponse is slower and smaller than in non pretreated nephrectomized rats B In a *nephrectomi ed* rat 300 µl of anti angiotensin changed the renin response (R) to a lower but still markedly in creased plateau Sensitivity to an giotensin is lowered to about 1 per cent of that found before the in jection of anti angiotensin C On ly in one out of 15 *nephrectomi ed* rats which received anti angiotensin at the plateau of the renin curve did the blood pressure reach its initial level the two doses of 100 µl of anti angiotensin each resulting in a stepwise fall to this level While the sensitivity to an giotensin is lowered the animal reacts with a reduced but typic ally prolonged response to a sec ond dose of renin A third dose of 100 µl of anti angiotensin now brings the blood pressure below the initial level D Contrary to the result shown in Fig 5 C this *nephrectomi ed* rat only responded with a marked depre sor effect to th first of four injections of anti angiotensin The blood pressure was low ered but still markedly above the starting level After the injections of anti angiotensin the rat was un able to respond to 20 times the dose of angiotensin which previously gave a distinct pressor response but it was still able to respond sl ghtly to renin

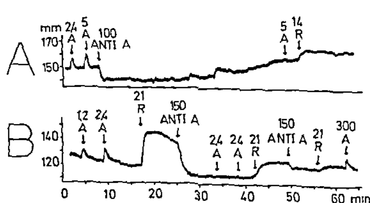


Fig 6 A A *ureterligated* rat responded with a depressor ef fect to 100 µl of anti angio ten in After the injection the rat did not respond to a dose of angiotensin which previously produced a marked pressor ef f ct but it was able to respond to renin with a pressor response which was 1) lower than found in non anti angiotensin pre treated ureterligated rats and 2) prolonged as found in r on pretreated ureterligated ani mals B A *ureterligated* rat re acted with rather small pressor responses to angiotensin and gave a prolonged response to renin A d se of 150 µl of anti angiotensin pro duced a rather steep fall to the initial level and the sensitivity to angiotensin decreased to less than 1 per cent The animal was still able to react with a prolonged response to renin but the response was smaller than that obtained before anti angiotensin was given A second dose of 150 µl of anti angiotensin caused only a slight decrease in blood pressure which stayed above the level it had at th time when the second dose of renin was injected

sure to the basic level when anti angiotensin was given shortly after the injection of renin (Fig 6 B) Even so, they were still able to react to a new injection of renin (Fig 6 B), the rise in blood pressure being more slow and less pronounced than that obtained before injection of anti angiotensin When they got a second injection of anti angiotensin the blood pressure fall differed from the first in that it did *not* reach the starting level This queer pattern was observed in all of 6 experiments in which ureterligated rats received a second injection of renin followed by a second injection of anti angiotensin (Fig 6 B)

5 *Continuously Angiotensin Infused Rats*

Ten normal and twelve nephrectomized rats were in 28 periods continuously infused intravenously with 9 ng of synthetic angiotensin II (Ciba) per minute *When the continuous injection was stopped* after a duration of about five to ten minutes the about 25 to 50 mm increased blood pressure would in most cases (20 of 28 periods) return to the level it had before the infusion (Fig 7 A) le in 4 tests it reached a 3 to 6 mm high vel and in the last 4 tests a level 11 to mm above the previous level *The effect 50 to 150 µl of anti angiotensin on the elevated blood pressure* by continuous angiotensin infusion was studied in five normal and sixteen nephrectomized rats In nine out of 21 rats the blood pressure returned to its initial level (Fig 7 A) in seven to 5 to 7 mm above this level while a level about 17 (11 12 15, 16, and 33) mm above the initial level was reached in five In the cases in which the blood pressure did not reach the initial level a second dose of anti angiotensin was without effect (Fig 7 B) Even when the infusion was stopped the blood pressure in these cases only returned to 6 and 8 mm above the initial level (Fig 7 B)

In six normal and 12 nephrectomized rats *anti angiotensin* was given shortly *before* a period of continuous infusion of 9 ng of angiotensin per min In seven experiments (six rats) the blood pressure did not respond but in 15 experiments (12 rats) there was a 14 mm (6–20 mm) increase in blood pressure, this increase being significant although markedly lower than the increase found in the same (and other) rats when untreated with anti angiotensin There was no difference between the responses of normal and nephrectomized rats Continuous infusion of the same volumes of physiological saline did not influence the blood pressure

6 *Rats with Acute Severe Construction of One or Both Renal Arteries*

24 to 48 hours after acute severe constriction of one or both renal arteries with 0 15 mm clips the initial blood pressure level and the response to 50 to 100 µl of anti angiotensin (K 765 A) of 9 anaesthetized rats were about the same as levels and responses found in anaesthetized normal rats although there was a tendency both to a more pronounced and a more steep blood pressure fall than in the normal rats (Fig 1 C and Fig 2 B as compared with the values in Fig 1 A and 2 A) Eight of the nine rats received repeated injections of anti angiotensin In all cases but one the second (or third) injection resulted in a markedly decreased depressor response (Fig 8 A) The injections caused an unresponsiveness to angiotensin equal to that in normal rats The response to renin was not tested

7 *Renal Hypertensive Rats*

Nearly all of the 20 renal hypertensive rats studied had a blood pressure of more than 200 mm Hg (175–240 mm) when measured by the tail pletysmograph method but somewhat lower values (150–220 mm Hg) when anaesthetized and measured with the mercury manometer The time interval between the clipping of the renal artery and the determination of the effect of anti angiotensin covered from three weeks to well over four months in most cases from 2 to

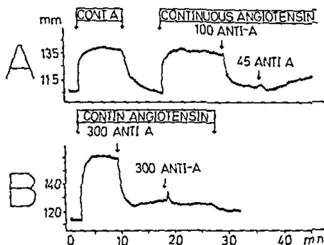


Fig 7 Effects of anti angiotensin on the increased blood pressure of rats *continuously infused with 9 ng of angiotensin per min* In Fig 7 A the anti angiotensin injection resulted in a steep blood pressure fall which was nearly identical with that obtained by cessation of the infusion Fig 7 B Injection of 300 µl of anti angiotensin (64 E) resulted in a decrease in blood pressure to a level markedly above the initial A second injection did not give further decrease Even when the infusion was stopped the initial level was not reached

4 months These differences in time seemed not to influence the results

All twenty renal hypertensive rats responded to a primary dose of 50 to 300 µl of anti angiotensin (K 765 A) with a steep depressor response which in many cases was more pronounced than that seen in normal rats (Fig 1 D and Fig 9 A D as compared with Fig 1 A and Fig 2 A) In nearly all rats the blood pressure returned to its initial level

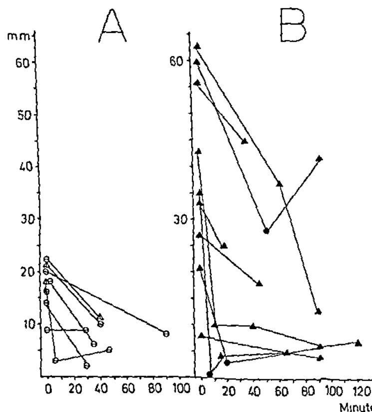


Fig 8 The figure shows the maximum depressor *effect of repeated doses of anti angiotensin* in mm Hg (ordinate) in A) rats with acute constriction of the renal arteries and B) renal hypertensive rats The primary depressor response is markedly more pronounced in the hypertensive rats In both groups the second and third doses result in much smaller respons s Abscissa Time in minutes after the first injection ⊖ and ● mark doses of 50 µl △ and ▲ doses of 100 µl

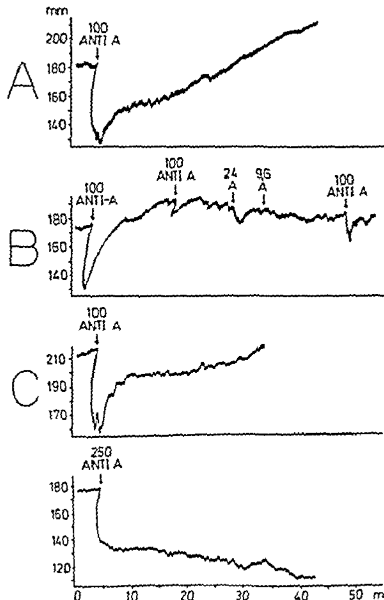


Fig 9 A B and C show the *steep and deep depressor effects of* anti angiotensin and the subsequent more or less rapid return to or above the high initial level of chronical renal hypertensive rats In one case (B) two more injections of anti angiotensin produced *depressions* of the blood pressure much smaller and shorter than the first Curve *D* shows the only lasting depressor effect of a single dose of anti angiotensin on a hypertensive rat.

in *from 5 to 60 minutes* Only one rat (Fig 9 D) reacted to an injection of anti angiotensin (250 µl) with a lasting depression

In nine hypertensive rats repeated injections of anti angiotensin were given at *intervals of at least 20 minutes* (Fig 8 B and 9 B) The response to subsequent injections was found to be less pronounced as in repeatedly anti angiotensin treated rats with acute severe constriction of one or both renal arteries (Fig 8 A) The blood pressure of seven of the hypertensive rats which received a total of about 300 µl (200–450 µl) of anti angiotensin returned to the starting level after each injection The blood pressure of one rat *returned to the* initial level after the first two injections of 100 and 50 µl but stayed low after the third (100 µl) which was given one hour and a half after the first One rat reacted to two doses of 150 µl in the same uncharacteristic way a steep fall with a return in less than 5 *minutes to* a value about 10 mm from the initial level followed by a secondary more slow fall

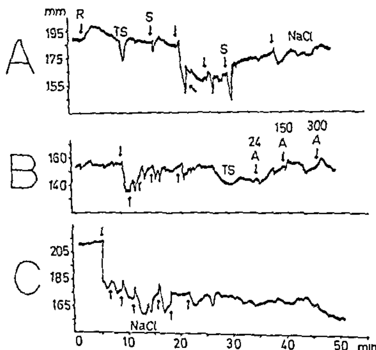


Fig 10 The effect of *repeated doses of anti angiotensin injected at intervals of a few minutes into* renal hypertensive rats In two rats (A and B) which received total amounts of 600 and 750 µl of anti angiotensin the blood pressure returned to its previous level In case C *in which the total injected volume was 900 µl the* pressure stayed lower but still at a hypertensive level In case A injection of normal rabbit serum (S) resulted in a small depressor followed by a small and short pressor response before injection of anti angiotensin and a short but marked depressor *response* after the *injection* Each unmarked arrow indicates injection of 150 µl of anti angiotensin Symbols see text to Fig 3

Eight of the hypertensive rats received *repeated injections* of 50 to 300 µl of anti angiotensin injected at *intervals of only about 2 minutes* the total amount of anti angiotensin injected per rat being about 600 µl (from 200 to 1050 µl) Again the previously found less pronounced response as regards magnitude of depression as well as duration was found (Fig 10) In five rats receiving a total dose of 200 300 600 750, and 800 µl respectively the blood pressure returned to or above the initial level in less than an hour (Fig 10 A and B), but in three rats receiving 500 650 and 1050 µl, respec

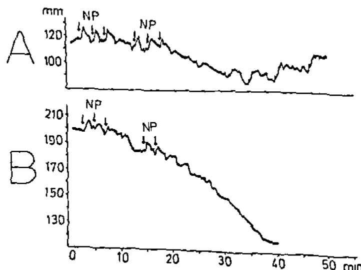


Fig 11 Effect of *repeated doses of* 150 µl of *normal rabbit plasma* (NP) injected at intervals of a *few minutes to a normal* (A) and to a hypertensive rat (B) In both rats the single injections caused a small and short *pressor response* but eventually they were followed by a fall in blood pressure which in the normal rat was about 30 *mm in the hypertensive* rat about 85 *mm It took about 2 hours* before the pressure *had returned* to the initial level

tively, the blood pressure stayed at the lower level or showed a further fall (Fig 10 C)

The effect of anti angiotensin on the sensitivity to angiotensin was about the same in hypertensive rats and in normal rats the sensitivity decreasing to less than 10 per cent and in some cases to about 1 per cent after injection of anti angiotensin Similarly the sensitivity to renin was decreased in the two cases in which it was tested

8 *Effects of Injection of Normal Rabbit Plasma*

In order to control the specificity of the effect of the anti angiotensin containing immune plasma from rabbits, normal rabbit plasma was injected into many of the rats in the different groups mentioned above When a single dose of 50 to 300 μl of normal plasma was injected, the rats in all groups responded with a short *pressor* response the blood pressure rising from a few to about 12 mm, the initial level being reached in less than 3 minutes If the rats had been pretreated with anti angiotensin they would most often react to normal plasma with the usual response, but a few of them reacted with a depressor response to normal plasma as well as to angiotensin and renin Repeated es of 150 μl of normal plasma given at rvals of only a few minutes resulted in ort pressor response but both in three normal (Fig 11 A) and in three hypertensive rats (Fig 11 B) the blood pressure would then fall to a low level from which it only slowly rose again

DISCUSSION

1 *Effect of Anti Angiotensin II on Blood Pressure*

The lack of effect of anti angiotensin on the blood pressure of *nephrectomized* rats is according to expectation and supports the belief in the specificity of the depressor action of the immune plasma on the other groups of rats The specificity is further supported by the lack of depressor action of plasma from normal rabbits on rats in these groups The varying degrees of the transient depressor response to anti angiotensin in normal *anaesthetized* rats (Fig 2 A) can partly be due to differences in plasma angiotensin and partly to differences in cardio-accelerator and pressor reflexes That the sensitivity of the baroreceptors plays a significant role is made highly probable by the lack of depressor effect in *conscious normal rats* (Fig 4) as it is known that barbiturates depress the compensatory reflexes (*Heymans* and *Neil* (1958)) The finding of a depressor effect of anti angiotensin in two of three *ergotamine tartrate pretreated conscious rats* is in accordance with this view, as ergotamine abolishes the baroreceptor reflexes (*Euler* and *Schmiterlo*w (1944)) The fact that sensitivity to angiotensin (tested before the anti angiotensin injection was given) is much lower in conscious than in ergotamine treated conscious rats and in anaesthetized rats (Fig 4) is a further sign of the correctness of this explanation These results make it highly probable that angiotensin plays a role for the blood pressure level of normal rats Because of the compensatory pressor reflexes this influence on the blood pressure is masked in conscious animals and only transiently visible in ergotamine treated conscious rats and in barbiturate anaesthetized rats

The depressor effect of anti angiotensin being relatively lower in *ureterligated* than in normal rats corresponds to the lowered sensitivity to injected angiotensin and renin which is found in ureterligated rats (*Bing* 1970) The relatively steep and deep depressor effect found in rats with *acute severe constriction of renal arteries* cannot be explained by an increased sensitivity of the vessels to angiotensin as this did not differ from that found in normal rats The difference can be due to increased plasma angiotensin

An increase in plasma angiotensin is also the most probable cause why the depressor

response to anti angiotensin is more pronounced in many *renal hypertensive* rats The finding that the depressor effect is only transient does so far not exclude a participation of angiotensin in the pathogenesis of renal hypertension as it has been shown that there is a resetting of the baroreceptor function at a higher pressure level in renal hypertensive animals (*Page* and *McCubbin* (1968)) The lack of a permanent depression of the blood pressure to a normal level could also be believed to be due to the use of too small doses of anti angiotensin but the very small further decrease in blood pressure obtainable by repeated injections (Fig 10 A and B) seems to exclude this possibility The permanent blood pressure fall found in some rats after rapidly repeated injections of anti angiotensin (Fig 10 C) must be taken as an unspecific reaction to the injected volumes of plasma as quite similar results were obtained in hypertensive rats injected with corresponding amounts of normal plasma (Fig 11 B)

II *Effect of Anti Angiotensin II on Sensitivity to Angiotensin and Renin*

A *Sensitivity to angiotensin* In previous experiments (*Hedwall* (1967 and 1968) and *Bing* and *Poulsen* (1968)) passive immunization with anti angiotensin was found to make rats unresponsive to injections of doses of angiotensin which before the injection of anti angiotensin resulted in pressor responses In the present study such decrease of sensitivity to angiotensin was found both in normal nephrectomized ureterligated and renal hypertensive rats (Fig 2 3 5 6 9 and 10) The sensitivity was in several cases reduced to less than 1 per cent The total duration of the decreased sensitivity was not determined as most experiments were shorter than two hours during which time the sensitivity was still low

B *Sensitivity to renin* As previously (1968) reported the effect of anti angiotensin on the sensitivity to renin is different in normal and nephrectomized rats In *normal* rats the response to renin is decreased so that doses which gave a marked pressor response before the injection of anti angiotensin are without or with only trifling pressor effect (Fig 3), the sensitivity being reduced to about 5 to 10 per cent Contrary to this anti angiotensin treated *nephrectomized* rats are still able to respond to renin with a prolonged pressor response which, however, is changed from the usual the rise being slower and less pronounced reaching maximum values of up to 50 per cent of those found in non pretreated nephrectomized rats When anti angiotensin was given to nephrectomized rats shortly *after* the injection of renin, the blood pressure would in all cases but one fall to a level which was markedly above the initial level even when doses of anti angiotensin were 10 times higher than those which could quickly lower the blood pressure to the initial level in renin treated normal rats (Fig 5 B and D and 3 B respectively) When nephrectomized rats received two renin injections one before and the other after injection of anti angiotensin, the pronounced changes both in type of rise and maximum increase were seen even when the second dose of renin was the double of the first (Fig 5 D) And a second dose of anti angiotensin would now only give a small decrease in blood pressure

The cause of this difference in effect of anti angiotensin on the sensitivity to renin in normal and nephrectomized rats is closely releated to the still unsettled question of the cause of the somewhat increased and markedly prolonged pressor response to renin which is found in nephrectomized animals The prolongation of the response would be easily understandable if the injected renin persisted for a longer time in the blood of the nephrectomised than in normal animals, but *Schaechtelin et al* (1964) found the half life of renin to be the same in normal and nephrectomized rats They therefore assumed that the prolonged effect could be due to an accumulation of renin in the arterioles of nephrectomized animals The results of the

present studies so far support this hypothesis as an accumulation of renin in the arterioles might be the reason for the only partial neutralization of the angiotensin If renin is deposited in the arterioles part of the new formed angiotensin may get into the circulation and thus be neutralized by the anti angiotensin while another part is released close to the arterioles and thus bound to their receptors, thereby escaping neutralization

This explanation is however made less probable by the finding that about half of the continuously angiotensin infused normal or nephrectomized rats reacted to anti angiotensin in the same way as renin pretreated nephrectomized rats the elevated blood pressure of both falling to a level significantly above the initial level In the other half of the continuously angiotensin infused rats anti angiotensin brought the blood pressure back to or close to the initial level which was also the result of anti angiotensin injection into renin pretreated normal and ureterligated rats The causes of these differences in response to anti angiotensin are unknown

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The effect of antiangiotensin on renal hypertension and on sensitivity to angiotensin has recently been studied by *A R Christlieb T L I Biber* & *R B Hickler* (J Clin Inv *48* 1506–1518 1969) by *I Eide* & *H Aars* (Nature 2?? 571 1969) and by *M Worcel P Meyer G Auriac* and *I Millie* (Plugers Arch *310* 251–263 1969)

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EPITHELIOID-CELL GRANULOMAS IN HODGKIN'S DISEASE

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The relevant literature on the occurrence of epithelioid-cell granulomas without a demonstrable infectious genesis in malignant systemic diseases—in particular Hodgkin s disease—is briefly reviewed Six cases of Hodgkin s disease with epithelioid-cell granulomas are reported and the aetiology discussed It is concluded that a consistent explanation of the phenomenon is not afforded by the material available up till now

Since 1934 there have been a few reports (Table 1) on patients with clinical and/or histological signs of sarcoidosis who have developed malignant systemic disease, either Hodgkin s disease (5 8 14 18) reticulo sarcoma (2 15), or lymphatic leukaemia (11) after follow up periods of less than 4 years These cases have been interpreted as malignant transformation of sarcoidosis (8 15) granulomatous reaction to malignant disease (11) coincidence of two different diseases (2 18) or atypical histological appearance of malignant lymphoma (5 14) Four of the patients had been receiving corticosteroids during the period prior to the verification of malignancy (5 8 11 15) resulting in all cases in distinct remission of the presumed sarcoidosis

Among 169 cases of Hodgkin s disease *Lennert & Mestdagh* (9) found 7 2 per cent to have a large content of epithelioid cells On the basis of supplementary biopsy material they set up a special clinical and histological variant of Hodgkin s disease which they called epitheloid zellige Lymphogranulomatose Histologically this type is characterized by massive infiltration by epithelioid cells arranged in small foci without forming granulomas Giant cells of the Langhans type were found in 30 per cent of these special cases

Bonenfant (1) also separated a special clinical histological variant of Hodgkin s disease lympho reticulose medullaire which he distinguished from paragranuloma In these cases too epithelioid cell hyperplasia was a predominant feature and Langhans giant cells were common However unlike *Lennert & Mestdagh Bonenfant* reported that the epithelioid cell hyperplasia might assume a granulomatous nature Both agreed that typical Reed Sternberg cells occurred in only a limited number of the cases

The type of Hodgkin s disease which *Lukes et al* (10) called lymphocytic predominance appears to be in several respects analogous to *Lennert & Mestdagh s* epitheloid zellige Lymphogranulomatose but *Lukes et al* did not report the occurrence of Langhans giant cells Furthermore they accept a case as Hodgkin s disease only if typical Reed Sternberg cells are demonstrable

Nickerson (13) mentioned that frequently

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he had observed sarcoid like lesions in Hodgkin's disease, but he did not describe these findings in detail

On the basis of the named studies it may be concluded that the signification of the occurrence of epithelioid cell granulomas in malignant systemic diseases—in particular Hodgkin's disease—is entirely obscure and that the frequency of this finding is unknown

Accordingly, it seemed of interest to publish the following cases which showed at the same time Hodgkin's disease and epithelioid cell granulomas with giant cells of the Langhans type *The histological classification of Hodgkin's disease was done according to the criteria used by Lukes et al* (10)

CASE REPORTS

Case 1 (Fig 1)

Male, *born 18 12 1915 Previously in good* health On 5 12 1958 he had a squamous-cell carcinoma of the lower lip treated with a radium implant No local recurrence 3½ years later lymph node enlargement in the left submandibular region without systemic symptoms On 27 3 1963 the tumour was excised and the lymph nodes in the carotid trigone were dissected The tumour showed Hodgkin's disease of the type mixed cellularity and the same lymph node contained epithelioid-cell granulomas with numerous giant *cells of the Langhans type many with Schaumann* bodies No signs of metastatic carcinoma Special staining for tubercle bacilli was not done No signs of Hodgkin's disease in other sites Postoperatively the patient had X ray irradiation to left side of the neck No signs of Hodgkin's disease since then In late 1967 he developed a cutaneous sarcoidosis like infiltration above the left ear and biopsy obtained on 19 1 1968 showed epithelioid-cell granulomas with a few giant cells No other cutaneous or mucosal infil*trations X ray examination revealed in the head* of the first metatarsal on the left a small punched out area There was still no enlargement of the lymph nodes or hepato splenomegaly and chest radiography still showed normal appearances Serological reactions for brucellosis and syphilis were negative The toxoplasmosis complement fixation reaction was negative while the Sabin Feldman dye test was positive in the dilution 1 50 The haematological status was unremarkable and serum electrophoresis serum creatinine liver func*tion tests and calcium metabolism were normal* Tuberculin or Kveim tests were not done

Case 2 (Fig 2)

Male born 27 7 1897 In 1963 chrysotherapy for mild rheumatoid arthritis of the hands and feet Otherwise he had been in good health In July 1967 swelling of lymph nodes in the left axilla and supraclavicular region without systemic *symptoms Biopsy from the left axilla on 20 7 1967* and from the left supraclavicular region on 23 8 1967 showed Hodgkin's disease of the type lymphocytic predominance Biopsy from a mediastinal lymph node on 15 8 1967 however showed epithelioid-cell granulomas with a few giant cells of the Langhans type but no signs of Hodgkin's disease Special staining for tubercle bacilli was not done The involved lymph node regions were irradiated with Co 60 In December 1967 lymph node enlargement was observed in the *right axilla and biopsy on 7 2 1968 showed* Hodgkin's disease of the type lymphocytic depletion Thereafter Co 60 radiation to the right axilla and the mediastinum Chest radiography and contrast lymphangiography showed normal appearances and no hepato-splenomegaly was demonstrable There were no signs of infiltrations in the skin mucous membranes or bones Haematological status unremarkable and serum electrophoresis liver function test serum creatinine and calcium metabolism normal Tuberculin and Kveim *tests negative, serological reactions for syphilis and* brucellosis were negative so was the toxoplasmosis complement fixation reaction and the Sabin Feldman dye test

Case 3 (Fig 3)

Female born 18 5 1927 Appendectomy in 1948 No pregnancies On 6 3 1960 total hysterectomy by the method of Wertheim because of a Stage I adenomatous cervical carcinoma In September 1961 enlargement of mediastinal lymph nodes was demonstrated in a routine chest radiography A lymph node biopsy from the left side of the neck on 1 11 1961 revealed Hodgkin's disease of the type nodular sclerosis From 1961 to 1964 repeated series of X ray irradiation to the neck and mediastinum but no signs of dis*semination* beyond these two regions In November 1964 he had developed a large tumour in the superior aperture of the thorax with bilateral recurrent nerve palsy After ibenzmethyzine medication some regre*ssion of the tumour But now* there had appeared in the large mediastinal tumour a cavity which communicated *with the* trachea The patient died *on 21 1 1965 s* Ilocated in the contents of the named cavity which had emptied out into the trachea Autopsy showed no signs of recurrence of the cervical cancer Hodgkin's disease of the type lymphocytic depletion was demonstrated in lymph nodes from

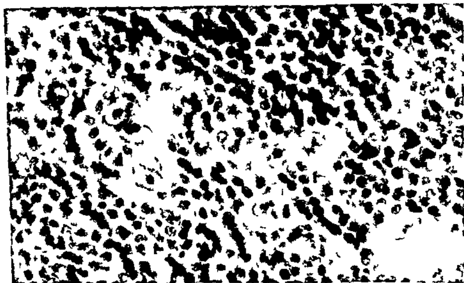


Fig 1a Hodgkin's disease— mixed cellularity



Fig 1b Epithelioid-cell granuloma with a giant cell of the Langhans type and a Schaumann body (a) and (b) are from the same left sided submandibular lymph node biopsy

the neck and mediastinum with invasion into the lungs trachea oesophagus and thyroid gland There were no signs of Hodgkin's disease below the diaphragm but epithelioid-cell granulomas were found in the spleen liver lumbar vertebral bone marrow – in the latter site with a *few giant* cells of the Langhans type No such *lesions were* observed above the diaphragm *The subdiaphragmatic* lymph nodes were not studied histologically but grossly they were not enlarged No infiltrations were found in the skin or mucous membranes The autopsy did *not include* any *study of skeletal changes A tuberculin test in* November 1964 had been negative *Special staining* showed no tubercle bacilli in the *autopsy preparations*

Case 4 (Fig 4)

Male born 27 8 1930 Previously in good health In February 1967 he developed tender swelling of lymph nodes in the left groin without *systemic symptoms Biopsy from the left groin* on 22 3 1967 showed *Hodgkin's disease of the type mixed cellularity* and large circumscribed *but not entirely well-defined granuloma like proliferations* of epithelioid cells with a few giant cells of the Langhans type Special staining for tubercle bacilli was not done Contrast lymphangiography revealed abnormal subdiaphragmatic lymph nodes but abnormal lymph nodes were not demonstrable above the diaphragm and chest radiography was normal No cutaneous infiltrations radiographic signs of skeletal infiltrations or hepato- splenomegaly Co 60 irradiation was administered to the involved subdiaphraematic lymph node regions and the patient has kept free of recurrence The haematological status was unremarkable and serum electrophoresis liver function tests serum creatinine and calcium metabolism were normal Serological reactions for [illegible]lis and brucellosis were negative The toxo[illegible] complement fixation reaction wa[illegible] nega[illegible] *but the* Sabin Feldman dye *test was positive* in the dilution 1 10 *A tuberculin test was not performed*

Case 5 (Fig 5)

Female born 6 7 1898 Para V Appendectomy in 193[illegible] Repeatedly ulcer of the right leg Her husband born 12 1? 1898 had been treated in April 1966 for Hodgkin's disease clinically in Stage II A histologically of the type mixed [illegible] For many years—perhaps more than [illegible] [illegible]atient herself had been aware of en[illegible]lar[illegible] lymph [illegible]odes on the neck and in the axilla In November 1966 she also noticed enlarged lymph nodes in both groins When seen in March 1967 she was *found* to have generalized lymph node enlargement and hepatomegaly later *splenomegaly was found* as well Numerous lymph *node biopsies* were obtained On 11 3 1967 *from the left groin on 21 3 1967 from the right groin* on 29 3 1967 *from* the right supraclavicular re*gion on 30 10 1967* from the right groin and *on 20 6 1968* from the right nuchal region as well as the mediastinum All the biopsy specimens showed the same *changes Obliterated lymph node structure with proliferation* of slightly poly*morphous somewhat immature lymphocytic* cells as well as *pronounced epithelioid-cell* proliferation *forming* numerous granulomas often containing *Langhans* giant cells and at times minor necroses No eosinophilia and very few plasma cells Typical Reed Sternberg cells were not demonstrable but there were scattered variants of Reed Sternberg cells Acid fast bacilli were not demonstrable by special staining Initially this case was *interpreted* as tuberculosis and from April 1967 anti tuberculous therapy was administered without effect for 7 months In November 1967 *she had X ray irradiation to the right groin where the* lymph node enlargement soon subsided The patient has not at any time had systemic symptoms *or other complaints on account of her disease* No cutaneous mucosal or skeletal changes were demonstrable Tuberculin test negative and no tubercle bacilli could be cultured from lymph node biopsies or urine Serological reactions for brucellosis and syphilis were negative In March 1967 serological reactions for toxoplasmosis were negative but one year later the Sabin Feldman dye test was positive *on 3 consecutive occasions in* 1 250 while the toxoplasmosis complement fixation reaction remained negative Chest X rays showed fibrous strands and calcifications as seen subsequent to tuberculosis and hilar node enlargement. Haematological studies showed constant mild anaemia and mild leucopenia with mild absolute lymphopenia while the platelet count was normal ESR *constantly around* 20 mm *Serum electrophoretic pattern,* liver function tests serum *creatinine* and calcium *metabolism normal*

Case 6 (Fig 6)

Female born *13 1 1928* Previously in good health Para III – in 1946 1949 and 1964 (twins) In September 1960 X ray examination done because of exertional dyspnoea showed severe pulmonary changes *consistent* with sarcoidosis Lymph node biopsy by the method of Daniel on 26 9 1960 showed typical epithelioid *cell granulomas with a few giant* cells of the Langhans type as well as a few foreign body giant cells No histological or clinical signs of Hodgkin's disease Special staining of the specimens for tubercle bacilli was not done Tubercle bacilli were not demonstrable on direct smear in the sputum or on culture of gastric *lavage* The

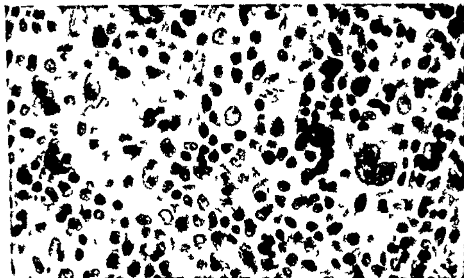


Fig 4a Hodgkin's disease— mixed cellularity



Fig 4b Not entirely well defined granuloma like epithelioid cell proliferations with a giant cell of the Langhans type (a) and (b) are from the same left sided iliac lymph node

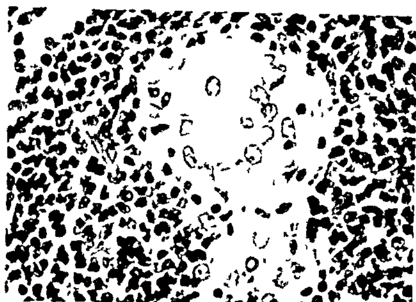


Fig 5a Epithelioid-cell granuloma with giant cell of the Langhans type

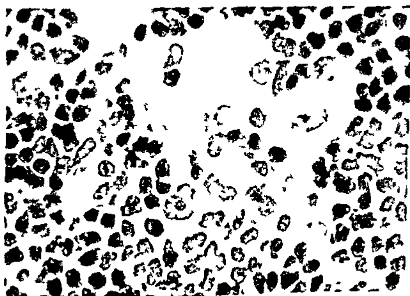


Fig 5b Reed Sternberg variant at the periphery of a small area with epithelioid cells



Fig 5c Two Reed Sternberg variants one of which is binuclear (a) – (c) are from different lymph nodes in the same patient

patient was tuberculin negative in spite of BCG vaccination in 1953 and 1956 Even though tuberculosis was thus very unlikely isoniazid was administered for 8 months during the first 6 months combined with Prednisone On this medication the infiltrative lesions in the lungs regressed considerably and the exertional dyspnoea decreased Thereafter the patient kept well in all essentials until June 1968 when she discovered an indolent intra abdominal tumour on a level with the umbilicus On 2 7 1968 a para aortic lymph node 8 cm in diameter on a level with L III was removed Enlarged lymph nodes were seen along the aorta up to the diaphragm The liver and spleen were normal Preparations of the tumour showed Hodgkin s disease of the type mixed cellularity but no signs of sarcoidosis No peripheral lymph node enlargement was palpable Mediastinoscopy on 29 7 1968 revealed hyperplastic lymph nodes and a biopsy from this site showed epithelioid cell hyperplasia with a tendency to granuloma formation but no signs of Hodgkin s disease Thereafter Co 60 irradiation to the subdiaphragmatic lymph nodes The patient did not exhibit any abnormal cutaneous or mucosal changes There were no abnormal skeletal changes of the spine or pelvis but hands and feet were not X rayed Chest radiography showed changes consistent with Stage III sarcoidosis Serological reactions for syphilis and brucellosis were negative The toxoplasmosis complement fixation reaction was negative but the Sabin Feldman dye test was positive in the dilution 1 50 Kveim s test was not performed In bronchial secretion no tubercle bacilli could be found on direct smear or culture The haematological status was unremarkable and serum electrophoresis liver function tests serum creatinine and calcium metabolism were normal

DISCUSSION

Epithelioid cell granulomas in the reticulo endothelial system may be observed not only in sarcoidosis but also in certain infectious diseases in particular tuberculosis but also in e g syphilis leprosy brucellosis histoplasmosis coccidioidomycosis and toxoplasmosis However none of the present 6 patients had a definite history clinical or serological evidence of an *infectious genesis* In the 4 cases where a tuberculin test was done it was negative but the value of this investigation is doubtful in Hodkin s disease as the patients are often anergic Only in one patient Case 5, did chest radiography suggest passed tuberculosis but repeated cultures from lymph node biopsies and urine yielded no growth of tubercle bacilli which were not either demonstrable in histological sections The patient was also tuberculin negative No patient showed serological signs of syphilis and in the 5 patients who had serological tests for

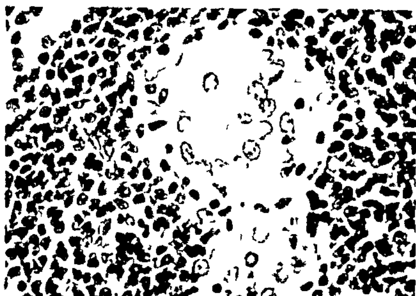


Fig 5a Epithelioid-cell granuloma with giant cell of the Langhans type

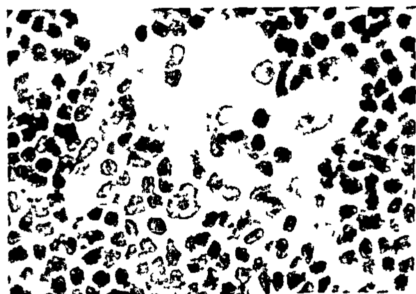


Fig 5b Reed Sternberg variant at the periphery of a small area with epithelioid cells

brucellosis the reactions were negative In 4 out of 5 cases thus studied the Sabin Feldman dye test was slightly positive but the toxoplasmosis complement fixation reaction was negative in all 5 cases

As an infectious genesis of the epithelioid cell granulomas in these 6 patients will be considered out of the question or at least extremely unlikely, there remain 5 possible explanations

A) Coincidence of sarcoidosis and Hodgkin s disease in the same patient

B) Transformation of sarcoidosis into Hodgkin s disease

C) The epithelioid cell granulomas may represent a nonspecific reaction to a malignant lymphoma in analogy to the changes which may at times be observed in lymph nodes draining an area housing a carcinoma (4 12)

D) The epithelioid cell granulomas may represent a particularly highly differentiated variety of the histiocytic epithelioid-cell proliferation found in Hodgkin s disease

E) If the formation of epithelioid cell granulomas in sarcoidosis is a consequence of the abnormal immunological state in this disease it may be imagined that occasionally the immunological disturbances in Hodgkin s disease may bear such a resemblance to the findings in sarcoidosis that they give rise to similar pathological changes

Re (A) Cases 1 and 6 fulfilled the histological and clinical criteria of sarcoidosis and both had Hodgkin s disease as well In these cases therefore possibility A is the most probable one Case 3 did not exhibit clinical signs of sarcoidosis but in this patient the epithelioid cell granulomas involved the liver spleen and bone marrow—*i e* organs which were not regional in relation to the mediastinal Hodgkin s disease Accordingly it is less likely that the epithelioid cell granulomas in this case represented a nonspecific regional reaction to a malignant disease and in this patient too there was perhaps also a question of a coincidence of sarcoidosis and Hodgkin s disease

Cases 1 and 3 had been treated for carcinoma However, this cannot be related to the occurrence of the epithelioid cell granulomas in Case 1 who had clinical sarcoidosis In Case 3, however it cannot be ruled out entirely that the previously treated malignancy may have played a role in the development of the epithelioid cell granulomas in the liver, spleen and bone marrow

Re (B) In principle, the possibility of a malignant transformation of sarcoidosis into Hodgkin s disease cannot be excluded in any of the 6 patients This possibility may apply in particular to Case 6 in whom 8 years elapsed from sarcoidosis was diagnosed until Hodgkin s disease was recognized Incidentally this patient like 4 out of the 8 cases in the literature had been treated with corticosteroids during the interval between the two diseases (5 8 11 15)

Re (C) In Cases 2 3 and 6 the epithelioid cell granulomas were not at the same site as Hodgkin s disease In Case 2 the epithelioid cell granulomas involved the mediastinum while Hodgkin s disease affected the axillae and the left supraclavicular region In Case 6 the sarcoidosis was mediastinal while Hodgkin s disease involved the abdominal lymph nodes In these two cases therefore there might be a possibility of a nonspecific regional reaction to a malignant lymphoma Case 3 has been discussed above

Re (D) In Cases 1 4 and 5 the epithelioid cell granulomas and Hodgkin s disease affected the same lymph nodes Case 1 has been discussed above, but it should be mentioned that although giant cells of the Langhans type were found in the epithelioid cell granulomas in all 6 cases Schaumann bodies were found in the giant cells only in Case 1 In Case 4 the epithelioid cell granulomas were not entirely well-defined and this might suggest a possibility of a particularly highly differentiated variety of the histiocytic epithelioid-cell proliferation occurring in Hodgkin s disease In Case 5 the epithelioid cell granulomas were well defined but the histiocytic epithelioid cells outside the actual granulomas were often either polymorphous and in these



Fig 1 Case 1 Longitudinal section Swollen yellow lesion with preserved muscle structure

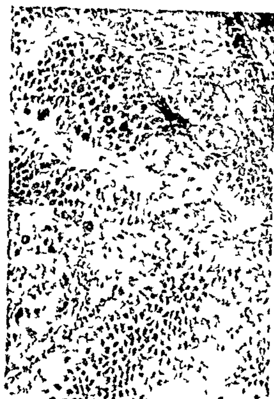


Fig 2 Case 1 Cross section showing loose connective septa with cellular infiltration van Gieson × 42



Fig 3 Case 1 Plump atypical cell in granulation tissue between preserved muscle fibers van Gieson × 680



Fig 4 Case 1 Large atypical *cells like ganglion* cells in granulation tissue van Gieson × 680

CONCLUSIONS

The findings in these cases agree well with those reported in other cases described under the name of proliferative myositis The condition is characterised by a short history of a tumour like lesion of the skeletal muscle with or without known preceding trauma The histological picture shows large polymorphous ganglioncell like elements which may be confused with malignant changes The condition is not malignant and shows no tendency to recur The histological picture is so characteristic that differential diagnosis will not offer difficulties when the lesion once has been seen For further information references is made to the publications cited

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THE PATHOLOGICAL-ANATOMICAL DIFFERENTIAL DIAGNOSIS BETWEEN MORBUS CROHN AND ULCERATIVE COLITIS

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One hundred and fourteen colon specimens were examined in order to analyse how often a number of previously defined pathological anatomical findings with relation to morbus Crohn and ulcerative colitis were present By different combinations of the histological findings the material was divided Groups based on wide and narrow definitions of the two diseases were made It was shown that a varying number of specimens could not fit into the groups and—if not very limited criteria were used—some specimens disclosed signs of both diseases When the macroscopic findings were related to the histological groups the number of specimens in the groups were always reduced Transmural inflammation an important sign of morbus Crohn was divided in two parts Focal lymphocyte collections and diffuse transmural inflammation It was demonstrated that *diffuse* transmural inflammation fits better with findings usually accepted as characteristics of ulcerative colitis than with characteristics of morbus Crohn A similar reasoning was carried out with a group of fissure shaped ulcers designated horizontal fissures It was suggested that transmural inflammation and fissure shaped ulcers may have been differently interpreted in the literature thereby being responsible for the reported differences in the ratio between morbus Crohn and ulcerative colitis If the occurence of granulomas transmural arranged focal lymphocyte collections and fistulas justify the diagnosis morbus Crohn this disease could be demonstrated in a smaller or larger part of the colon in 29 out of the 114 patients (25 per cent)

The realization that Crohn s disease (Mb Crohn) primarily conceived as a disorder solely affecting the terminal ileum (*Crohn et al* 1932), may extend to the colon (*Colp* 1934) or occur as an isolated lesion here (*Wells* 1952) has caused differential diagnostic problems as regards ulcerative colitis

Both diseases are unspecific inflammations and the possibility that they are different manifestations of a common aetiological mechanism cannot be absolutely rejected On the other hand clinical radiological and pathological anatomical differences have—to most investigators—justified a distinction between them

In recent years a large number of publications have dealt with the problems (survey *Axelsson & Johansen* 1970) The conclusions of these studies have been that the typical pathological findings of *Mb Crohn* are a thick intestinal wall sometimes with a cobble stone relief and histologically revealing inflammation—with or without granulomas—through all layers of the intestine fissure

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shaped ulcers and oedema In *ulcerative colitis* the intestinal wall is often thin granulated with extensive ulcerations and pseudopolyp formation Histologically the inflammation and the ulcerations are superficial and crypt abscesses and hyperaemia are often seen

In the major part of the publications the pathology is described on the basis of two groups of patients or specimens without revealing what primarily was demanded for placing the patient or the specimen in one of the groups Furthermore the findings are sometimes badly defined and the frequency and extension with which they occur is not always given

We intend to approach the subject from an other angle In revising the colon specimens from our department our chief purpose will be to answer the question What was really seen or found in the individual specimens and later—by different combinations of these findings—we will explore in which way the material may be distributed between the two diseases

No clinical or radiological information has been procured and no attention has been paid to such information if available

MATERIAL AND METHODS

One hundred and twenty nine *colon specimens* all resected on account of inflammatory disease in the years 1959–1968 were examined Fifteen were considered insufficiently sectioned the remaining 114 constitute the material The last resected 39 specimens were primarily treated by one of the authors Photographs from all these specimens were prepared and an average of 32 sections were taken from each colon In the rest of the material 18 blocks per specimen together with some photographs were available In a few cases it was necessary to supplement the macroscopic description by the pathologist with that by the surgeon

The specimens were fixed in formalin Staining reactions were haematoxylin-eosin and van Gieson Occasionally the PAS reaction and different elastin stainings were used The granulomas were explored in polarized light

All the specimens were examined by each of the authors separately and the findings recorded on punched cards The results were nearly identical During a third examination the few differences were discussed and eliminated and this set of punched cards became the definitive one

NOMENCLATURE

Before giving the results a comment should be given on some of the findings which are not self evident

Macroscopic Findings

All specimens recorded as ***toxic megacolon*** had when they were opened a maximum circumference of more than 18 cm and were extremely thin walled

The mucosal relief designated as ***granulated*** was built up of smaller and larger jags or teeth situated close to each other Sometimes the jags had taken the character of anastomosing ridges giving the mucosa an appearance like a trimmed poodle When the mucosal jags were large—one centimeter or more—so that they projected into the lumen like polypoid structures they were registered as ***pseudopolyps***

Cobblestone was a mucosal pattern composed of oedematous polygonal mucosal pads standing up between deep cleft formed ulcerations

Concerning the ulcers ***large irregular ulcers*** with an archipelagolike appearance were separated from more ***regular longitudinal ulcers*** running in the direction of the intestine All other ulcers were designated ***uncharacteristic***

An ***abnormal serosa*** signified that the serosa was thickened and indurated with signs of adhesions and sometimes remnants of fistulas When the lymph nodes were enlarged and consequently seemed to be present in an increased number they were recorded as ***conspicuous lymph nodes***

Histological Findings

Among the histological findings ***granulomas*** and ***crypt abscesses*** were registered as ***many*** when several were found in every section and as ***few*** when only one or two were found in some of the sections Only convincing granulomas of the sarcoid type consisting of epitheloid cells and giant cells of the Langhans type without central necrosis were recorded

Transmural inflammation was defined as an inflammation through all layers of the intestinal wall including the subserosa and the serosa An attempt was made to separate two different types 1) One in which ***focal collections of lymphocytes***—often perivascularly distributed—was the dominant finding (Fig 1) The interjacent inflammation was inconspicuous In extensive cases such slides had a spotted appearance when viewed macroscopically 2) ***A diffuse type*** (Fig 2) where such collections sometimes were seen but never predominated In this type the inflammatory exudate was often more pleomorph and contained besides lymphocytes,



Fig. 1. Focal lymphocyte collections transmural arranged. No granulomas were present. A. Survey. A small cleft is seen (H & E × 6). B. (H & E × 25)



Fig. 2. Diffuse transmural inflammation. Tunica muscularis is illustrated (v. Gieson × 40)



Fig 3 A fistula (H & E × 10)
Fig 4 A A horizontal fissue (H & E × 25) B A horizontal fissue coming out from the angle of a superficial ulceration (H & E × 40)

plasma cells histiocytes and granulocytes Even very slight degrees of this diffuse type of inflammation we e recorded.

Histolog cally the ulcers were registered in two main groups 1) *Superficial ulcers* were flat and limited to the mucosa and the submucosa and 2) *Fissure shaped ulcers* which again were sub divided into three sections a As *regular fistulas* were regi tered long narrow ulcers extending through the whole of the intestinal wall (Fig 3) b *Clefts* were sl ghtly broader and only ext nded down in the mi scularis These two sorts of fissures often had a direction perpendicular to the mucosa c The third section was marked *hori ontal fissures* referring to their horizontal or oblique course (Fig 4) Fissures coming from the angles of superficial ulcers were recorded in this group

The mucosa between the ulc rs was *intact* when the crypts had preserved their perpendicular narrow appearance and *deformed* when they appeared tortuous anastomosing running in all directions An inflammatory exudate could be present in both types of mucosa

Reduced mucin secretion was seen wh n the epith lial destruction had caused loss of goblet cells and corresponding impairment of the amount of mucin secretion *Neuromatous hyperplasia* was registered when the ganglion cells were prominent or when neuromalike structures were found in the submucosa

RESULTS

The punched cards permits a large number of different combinations of the findings Below these findings are correlated not according to any predetermined principle but in a way

TABLE 1 *Frequency of the Macroscopic Findings in 114 Colonspecimens*

Thick intestinal wall	51 (45 %)
Thin intestinal wall	14 (12 %)
Toxic megacolon	6 (5 %)
Strictures	20 (18 %)
Granulated mucosa	71 (62 %)
Pseudopolyps	67 (59 %)
Cobblestone relief	12 (11 %)
Large irregular ulcers	36 (32 %)
Long regular ulcers	19 (17 %)
Uncharacteristic ulcers	39 (34 %)
No ulcers	10 (9 %)
Abnormal serosa	23 (20 %)
Conspicuous lymphnodes	26 (23 %)

TABLE 2 *Frequency of the Histological Findings in 114 Colonspecimens*

Sarcoid granulomas	many	11 (10 %)
	few	8 (7 %)
Crypt abscesses	many	65 (57 %)
	few	31 (27 %)
Transmural inflammation	diffuse	61 (54 %)
	focal	25 (22 %)
Fissure shaped ulcers	fistulas	8 (7 %)
	clefts	24 (21 %)
	horizontal	46 (40 %)
Superficial ulcers		63 (55 %)
Submucosa mainly	fibrous	83 (73 %)
	oedematous	28 (25 %)
Mucosa between ulcers	deformed	78 (68 %)
	intact	36 (32 %)
Hyperaemic mucosa and submucosa		86 (75 %)
Eosinophilia	conspicuous	15 (13 %)
	inconspicuous	65 (57 %)
Paneth cell metaplasia		31 (27 %)
Pseudopyloric glands		4 (4 %)
Neurogenic hyperplasia		11 (10 %)
Reduced mucin secretion		55 (48 %)

which to us seems practical All the microscopic slides were available for reexamination and discussion and therefore histological criterions more than macroscopic findings will be the basis for the classification

The incidence of a series of macroscopic and histological findings are summarized in Tables 1 and 2 Tables 3 and 4 are concerned with findings related to *Mb Crohn* Table 3 demonstrates that although transmural inflammation most often is seen in sections with granulomas the diffuse form is present in more than half of the sections without A group of specimens composed of both those with granulomas and those with transmural inflammation without granulomas will be reduced by more than 50 per cent if the diffuse type of transmural inflammation is extracted A group containing the specimens *disclosing either granulomas or focal lymphocyte collections* is made It comprises 29 *specimens Below this group will be looked* upon as a unity and it will never be divided *The diffuse form of transmural inflammation* will be treated later

The fissure shaped ulcers are a frequent finding in this above mentioned group—granulomas or focal lymphocyte collections (Table 4) All fistulas are found here Horizontal fissures which will be dealt with later are an exception since they are more commonly found in sections without granulomas or focal lymphocyte collections

TABLE 3 *The relation between Granulomas and Transmural Inflammation The Transmural Inflammation is Divided in Two Forms Focal Lymphocyte Collections and Diffuse Inflammation*

		Transmural inflammation		
		Focal	Diffuse	Either focal or diffuse
Specimens total	114	25 (22 %)	61 (54 %)	[illegible] (64 %)
With granulomas	19 (17 %)	15 (79 %)	10 (53 %)	18 (95 %)
Without granulomas	95 (83 %)	10 (11 %)	51 (54 %)	54 (57 %)

Specimens with either granulomas (19) or transmural inflammation *without granulomas* (54) = 73 specimens

Specimens with either granulomas (19) or *focal* lympocyte collections without granulomas (10) = 29 specimens

TABLE 4 *The Relation between the Group Granulomas or Focal Lymphocyte Collections and the Different Forms of Fissure Shaped Ulcers*

		Fistulas	Clefts	Horizontal *fissures*	Either fistulas or clefts	Either fistulas or clefts or horizontal fissures
Specimens total	114	8 (7 %)	25 (22 %)	46 (40 %)	28 (25 %)	67 (58 %)
Either with granulomas or focal lymphocyte collections	29 (25 %)	8 (28 %)	16 (55 %)	11 (38 %)	19 (66 %)	27 (93 %)
Without granulomas or focal lymphocyte collections	85 (75 %)	0 (0 %)	9 (11 %)	35 (41 %)	9 (11 %)	40 (47 %)

Specimens with either granulomas or focal lymphocyte collections (29) or fistulas or clefts or horizontal fissures without those findings (40) = 69 specimens

Specimens with either granulomas or focal lymphocyte collections (29) or fistulas or clefts without those findings (9) – 38 specimens

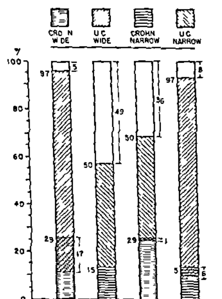


Fig 5 The combination of the histologically defined groups (wide and narrow) of mb Crohn and ulcerative colitis expressed in percentage Left to the collums are placed the total number of specimens in the wide and narrow groups Right to are placed the number of unclassified and overlapping specimens

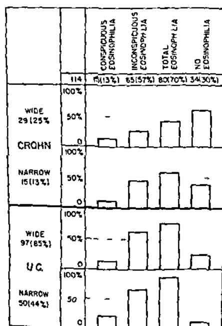


Fig 6 The distribution of tissue eosinophilia in the two groups (wide and narrow) of mb Crohn and ulcerative colitis expressed in percentages

TABLE 5 *The Relation between the Group Both Granulomas or Focal Lymphocyte Collections and Fistulas or Clefts and the State of the Mucosa*

		Mucosa between ulcers	
		Intact	Deformed
Specimens total	114	36 (32 %)	78 (68 %)
With both granulomas or focal lymphocyte collections and fistulas or clefts	19 (17 %)	15 (79 %)	4 (21 %)
Without granulomas or focal lymphocyte collections and with no fistulas or clefts	76 (67 %)	13 (17 %)	63 (83 %)

TABLE 6 *Four Different Definitions of Mb Crohn Based on the Occurrence of Histological Findings*

1	Granulomas	19 (17 %)
2	Granulomas or focal lymphocyte-collections	29 (25 %)
3	Granulomas or focal lymphocyte collections and fistulas or clefts	19 (17 %)
4	Granulomas or focal lymphocyte collections and fistulas or clefts and intact mucosa between the ulcers	15 (13 %)

By including the state of the mucosa between the ulcers the groups are narrowed even more (Table 5)

As mentioned, the applied findings are generally accepted as characteristics of *Mb Crohn* In Table 6 four possible definitions of the disease based on these microscopic findings are presented Group 2 is the most extensive group 4 the most limited Below they will be designated Crohn wide and Crohn narrow respectively

The following tables are concerned with findings often related to *ulcerative colitis* Table 7 demonstrates that only one specimen without crypt abscesses shows heavy hyperaemia Since the occurrence of crypt abscesses at all is a very wide characteristic it was decided to narrow the group and to continue only with the group Many crypt abscesses In Table 8 these are crossed with superficial ulcerations and the combined results are related to the state of the mucosa in Table 9

In Table 10 are presented again four sets of findings this time used as histological definitions of ulcerative colitis Group 2 will be designated u c wide and group 4 u c narrow

The combination of wide and narrow definitions is given in Fig 5 When the wide definitions are connected only [illegible] specimens do not fit into the microscopic classification

Table 7 *The Relation between Crypt abscesses and Hyperaemia of the Mucosa and Submucosa*

		Heavy hyperaemia	No or slight hyperaemia
Specimens total	114	86 (75 %)	28 (25 %)
With many cryptabscesses	65 (57 %)	62 (95 %)	3 (5 %)
With few cryptabscesses	31 (27 %)	23 (74 %)	8 (6 %)
With cryptabscesses *total*	96 (84 %)	85 (89 %)	11 (11 %)
With no cryptabscesses	18 (16 %)	1 (6 %)	17 (94 %)

Specimens with either crypt abscesses (96) or heavy hyperaemia without crypt abscesses (1) = 97 specimens

TABLE 8 *The Relation between Many Crypt abscesses and Superficial Ulcerations*

		Superficial ulcerations	No superficial ulcerations
Specimens total	114	63 (55 %)	51 (45 %)
Many crypt abscesses	65 (57 %)	51 (78 %)	14 (27 %)
Few or no crypt abscesses	49 (43 %)	12 (24 %)	37 (76 %)

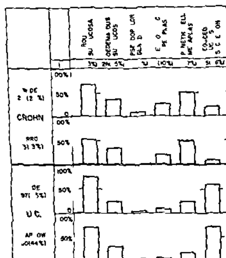


Fig 7 The distribution of six histological findings in the two groups (wide and narrow) of mb Crohn and ulcerative colitis expressed in percentages

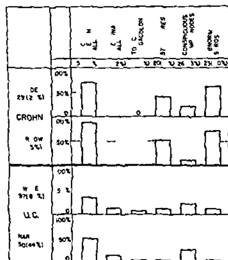


Fig 8 The distribution of six macroscopic findings in the two groups (wide and narrow) of mb Crohn and ulcerative colitis expressed in percentages

On the other hand 17 fulfil all criterions for both diseases No such specimens were found when the narrow definitions were used but 49 specimens remained unclassified

In the following the wide and narrow groups are related to other histological and macroscopical findings Fig 6 demonstrates that tissue eosinophilia *is most frequent in* ulcerative colitis but also occurs in several specimens with Mb Crohn Fig 7 shows that other histological findings are nearly all represented in the four groups and thus limit the criterions to a variable degree

Figs 8 and 9 are concerned with macroscopic findings Thin intestinal wall and toxic megacolon are only found in ulcerative colitis *It is* noteworthy that conspicuous lymph nodes are found more often in ulcerative colitis than in Mb Crohn The cobblestone relief is found in a few specimens with ulcerative colitis but in a third of the specimens with Mb Crohn

Fig 10 is dealing especially with transmural diffuse inflammation This phenomenon granulomas and focal lymphocyte collections are related to findings previously used as criterions for the two diseases It is seen that the group pure transmural diffuse inflammation *contains more characteristics* of ulcerative colitis than of Mb Crohn

Calculations concerning horizontal fissures are carried out in exacly the same way in Fig 11

At last the localization of the diseases in

TABLF 9 *The Relation between the Group Many Crypt abscesses and Superficial Ulcerations and the State of the Mucosa*

		Mucosa between ulcers	
		Deformed	Intact
Specimens total	114	78 (68 %)	36 (32 %)
Many crypt abscesses superficial ulcerations	51 (45 %)	50 (98 %)	1 (2 %)
Few or no crypt abscesses no superficial ulcerations	37 (3? %)	10 (27 %)	27 (73 %)

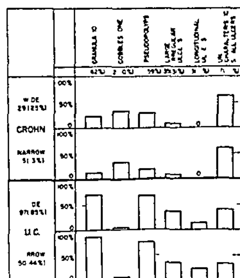


Fig 9 The distribution of the muco al patterns and ulcers in the two groups (wide and narrow) of Crohn and ulcerative colitis expressed in per

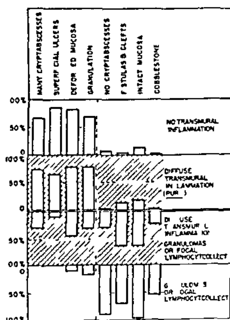


Fig 10 The relation between transmural inflammation especially the diffuse form and four characteristics of mb Crohn and of ulcerative colitis expressed in percentages

TABLE 10 *Four Different Definitions of Ulcerative Colitis Based on the Occurrence of Histological Findings*

1	Crypt abscesses	96 (84 %)
2	Crypt abscesses or hyperaemia	97 (85 %)
3	Many crypt abscesses and superficial ulcerations	51 (45 %)
4	Many crypt abscesses and superficial ulcerations and deformed mucosa between the ulcers	50 (44 %)

relation to the given histological definition is illustrated in Fig 12 Concerning the 29 specimens in the Crohn group 19 were colectomy specimens the remaining 10 consisted only of the ileum coecum and colon ascendens The localization of the disease is given by indicating the part of the bowel which was normal Ulcerative colitis specimens are treated in the same way 86 were colectomy specimens The most common part of the colon represented was the sigmoideum

Pertinent to the localization but not given

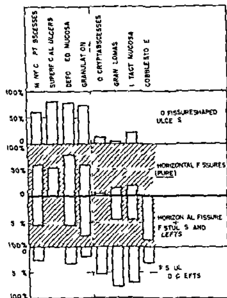


Fig 11 The relation between fissure shaped ulcers especially horizontal fissures and four characteris tics of mb Crohn and of ulcerative colitis expressed in percentages

in the figures is that the disease in four spe cimens was segmentally distributed Three of them revealed a histological picture placing them in the Crohn narrow group The last one belongs in the groups of ulcerative colitis

Concerning anal lesions 5 of the Crohn specimens disclosed granulomas in the sec tions from anus

At last it should be added that in the 19 specimens with granulomas in the intestinal wall 10 had granulomas in the regional lymph nodes as well

DISCUSSION

Where a number of colon specimens had to be classified between Mb Crohn and ulcera tive colitis it is of utmost importance that the classification is based on criterions for each disease, and not on a principle for elimi nation Especially when reviewing a series of previously diagnosticated specimens this last mentioned principle could be tempting

The applied findings had to be well de fined In this work ome of the definitions are rather extensive and estimation are used instead of countings imitating the daily rou tine as close as possible

The occurrence of the two diseases in rela tion to each other is reported with varying frequency According to several authors (*Cornes & Stecher* 1961 *Gjone* 1967 *Maratka et al* 1968) ulcerative colitis is 10–15 times more frequent in the colon than Mb Crohn However some others e g (*Monk et al* 1967) indicate a ratio about 50 per cent There is general agreement that—in addition to the granulomas—transmural inflammation and fissure shaped ulcers are the most distinctive findings of Mb Crohn (*Rappaport et al* 1951 *Lockhart Mummery & Morson* 1960 *Williams* 1964 *Farmer et al* 1968) The pathological interpretation of these findings may be one of the reasons responsible for such a difference

By dividing the group transmural inflam mation into two parts (Table 3) it is con spicuous that focal lymphocyte collections are often found together with granulomas

IN E NAL SEG E TS A LA LE OR EXA IQ	25(3)	9(6)					0 1
		29(5)	2 (5)	13(6)	19(6)	(6)	
CROHN DE 29 (NA OW 5)	LE	COECUM	SCE D	TR S	DESCEND	SIG D	ECTU
NO L	8(6)	5(3)	3(2)	2( )	(0)	2(0)	1( )

NTES INA SEG E TS AV ABLE FOR EXAMINATIO	77(41)	86(50)					5(2)
		88(50)	8 (50)	86 5)	85(50)	97(50)	
U C. W DE 97 (N RROW 50)	LEUM	COECU	S E D	TR SV	DES END	S GMOID	RECT
ORMAL	3 (5)	26( )	(7)	4( )	0(0)	2(2)	0(0)

Fig 12 The distribution of the two histologically defined groups (wide and narrow—narrow in brackets) of mb Crohn and ulcerative colitis in the intestinal tract The different seg ments available for examination are indicated Segments without patholo gical findings are shown downward in the figures

and that the *diffuse transmural inflammation* is more common in specimens without granulomas There is a morphogocical similarity between granulomas and focal lymphocyte collections, and not seldom granulomas are seen to develop in the reaction centres of lymphocyte collections The fact that the diffuse inflammation especially in its pure form combined more cogently with the characteristics of ulcerative colitis than with those of Mb Crohn, (Fig 10), may support the view that diffuse transmural inflammation is a ulcerative colitis sign Here again it has to be stressed that even the slightest degrees of diffuse *transmural inflammation* were registered It is our impression that when transmural inflammation attains severe degrees focal lymphoid hyperplasia nearly always occurs An exception from this are specimens with toxic megacolon where diffuse inflammation without any signs of lymphocyte collections may be severe

The reason for separating what we designate horizontal fissures from other fissure shaped ulcers was primarily that small but sometimes even longer fissures often extended out from the angles of typical superficial ulcerations undermining the mucosa Yet they are also found without relation to superficial ulcerations In our opinion they are fundamentally different from fistulas and clefts which nearly always have a direction perpendicular to the mucosa and have no connection with superficial ulcerations

If diffuse transmural inflammation and horizontal fissures defined in the above mentioned way are looked upon as criterions of *Mb Crohn* about half of the specimens would be classified as Crohn specimens

The tables concerning ulcerative colitis are dominated by the extreme unspecific nature of the single findings Hyperaemia crypt abscesses and superficial ulcerations can be found in a large number of diseases Superficial inflammation may have been used as a criterion for ulcerative colitis but since we have registered 72 specimens (Table 3) with one or other form of transmural inflammation the maximum number of such specimens would only have been 42 Therefore we have preferred to indicate the superficial nature of ulcerative colitis by recording superficial ulcerations and crypt abscesses

The percentage combination of the wide and narrow criterions of Mb Crohn and ulcerative colitis discloses two important groups of specimens (Fig 5) An unclassified group and an overlapping group The former varies from 4 to 43 per cent Especially when the narrow criterions of ulcerative colitis are used *the unclassified group occupies a considerable* number The seventeen specimens (15 per cent) revealing findings common to both diseases when the wide definitions are used disappear completely when the narrow ones are employed

In a recently published work concerning the differential diagnosis between Mb Crohn and ulcerative colitis and the related terminology *Riis & Anthonisen* (1969) have proposed that patients with ulcerative colitis disclosing signs of Mb Crohn too should be designated Ulcerative colitis Crohn positive patients This may apply to the overlapping groups in this pathological study Although all the findings are unspecific many still believe that the criterions of Mb Crohn are a little more fixed than those of ulcerative colitis and therefore it might be reasonable to turn round and designate these overlapping specimens Crohn ulcerative colitis positive specimens

A reduction nearly always takes place when the groups are combined with other findings The tissue eosinophilia estimated as a total is in good accordance with the results obtained by *Riis & Anthonisen* (1964) yet it is remarkable that conspicuous eosinophilia is practically the same in ulcerative colitis and *Mb Crohn*

Concerning the state of submucosa (Fig 6) no clear predominance of fibrosis or oedema is *demonstrated Although even* light degrees of fibrosis are registered the number is surprisingly high We can state that the two signs were *relatively difficult to register* since fibrosis and oedema varied from slide to slide in the same specimen

Pseudopyloric glands and neurogenic abnormalities are findings too rare to allow any conclusions

The more frequent occurrence of Paneth cells in Mb Crohn than in ulcerative colitis is not in agreement with other investigators (*Lennard Jones et al* 1968) Paneth cells are present in the colon especially when regeneration takes place, and this is supposed to be more common in ulcerative colitis than in Mb Crohn A reduced mucous secretion most often found in ulcerative colitis is in good accordance with the literature (*Hellstrom* & *Fisher* 1967)

By incorporating the macroscopic findings the expected results were obtained In this series no Crohn specimens disclosed toxic megacolon though such cases have been reported (*Hauk et al* 1967)

It is remarkable that conspicuous lymph nodes were present more often in the specimens with ulcerative colitis than in those with Mb Crohn Several authors stressed the opposite view (*Rappaport et al* 1951 *van Patter et al* 1954, *Farmer et al* 1968), but some (*Cornes* & *Stecher* 1961 *Brooke* 1959) had made the same observation Concerning the mucosal relief it is noted that a considerable number of specimens in the Crohn groups disclosed granulations This was indeed the case but it may be interposed that the demand on cobblestones was very narrow and some of the specimens may have disclosed this appearance in a modified form On the other hand a few of the specimens in the ulcerative colitis group discloses cobblestone relief an observation also made by others (*Goldgraber* 1965)

This investigation has not answered the question How many specimens were virtually Crohn specimens and how many were ulcerative colitis specimens Nor was this intended But by using a series of reproducable findings it has been shown that 1) The size of the groups formed by the combinations vary considerably that 2) A group of unclassified specimens always appear and that 3) The groups intermingle with each other if not very limited criterions are used

We are well aware that other findings and other calculations could have been used yet we feel sure that the results would principally have been the same

In daily practice we intend to use the wide criterions for Mb Crohn This means that all specimens with granulomas or transmural lymphocyte collections will be diagnosed as such As mentioned all slides containing fistulas were contained in this group (Table 4) and this finding will also strongly support the diagnosis Diffuse transmural inflammation and horizontal fissures will as solitary findings not provoke the diagnosis

Ulcerative colitis is in some ways more difficult to interpret It is obvious that the wide group contains too many specimens at any rate the Crohn positive The narrow group rules out all cases demonstrating the disease in more chronic forms It might be necessary to reduce the wide group—containing slides with even few crypt abscesses—with histological findings other than those used here or by macroscopic signs

In this connection it has to be remembered and stressed that this investigation is concerned with specimens not patients The final diagnosis of the patient—if it is possible to make it—is composed of anamnestic rectoscopic radiological and maybe even cytological and immunological facts in combination with the pathological anatomical diagnosis It is our impression that the pathology contributes more to the diagnosis of Mb Crohn than to ulcerative colitis

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THE EFFECT OF HIGH DOSES OF FLUORIDE ON DISUSE ATROPHY OF BONE IN YOUNG RATS

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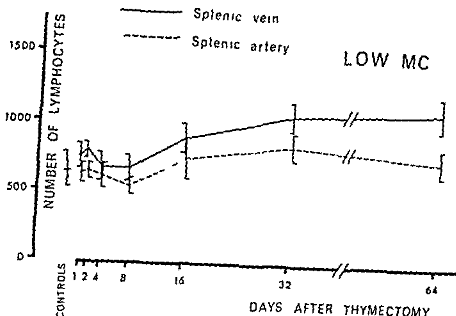
Male weanling rats were supplied a standard mixed diet (SIFF Norsk Standard) and were given tap water containing 0 02 ppm of F (control group) or water containing 45 ppm of F (fluoridated group) for 4 weeks At this time the right hind leg of all animals was paralysed by severing the sciatic and femoral nerve The rats were then continued on the same food and water regimen for 3 weeks The specific gravity ash weight and calcium content of the dry defatted femora were measured The differences in ash and calcium content in the paralysed and the normal legs were found to be similar in both groups In the fluoridated animals however the normal limbs showed significantly higher mean values of ash content and specific gravity than the corresponding legs of the control animals The mean ash content of the paralysed limbs of the fluoridated animals was slightly higher and the mean calcium content and specific gravity were similar to the values observed in the normal limbs of the control animals Apparently the high fluoride intake was associated with an increased rate of bone formation eliminating the effects of disuse atrophy of bone in the paralysed femora

In recent years the effect of high doses of fluoride on bone tissue has attracted considerable attention (9 22) In adult human subjects a relatively high content of fluoride in the drinking water seems to be related to reduced incidence of osteoporotic changes (4 12) In the treatment of several diseases accompanied by osteoporosis very high doses of fluoride have been employed (18 19 20) Most reports indicate a favorable effect from doses of 30 to 100 mg of F per day for periods up to 34 months (1 3 5 18 19 20) although there is some diversity of opinion as to the value of such treatment (14)

Large numbers of investigations on the effects of high doses of fluoride on bone tissue in various experimental animals have been published Only few animal studies however are concerned with the possible preventive effect of fluoride on experimental osteoporosis and the results are not entirely in agreement *Gedalia et al* (10) reported that 25 ppm of fluoride in the drinking water given over a 2 months experimental period counteracted the development of osteoporosis of femora caused by disuse atrophy of para

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Requests for reprints should be addressed to Dr Gisle Bang the Gade Institute Department of Pathology University of Bergen Norway



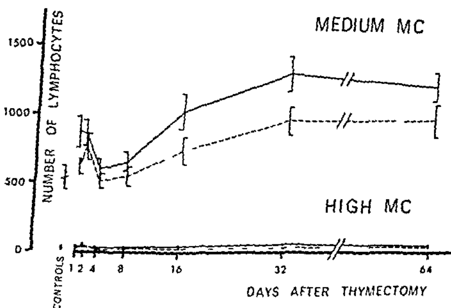


Fig 2

Number of lymphocytes per mm³ of blood from a splenic vein and a splenic artery at different intervals after thymectomy The lympho ytes are sub livided into cells with low medium and high mitochondrial content (MC) Mean ± S E.

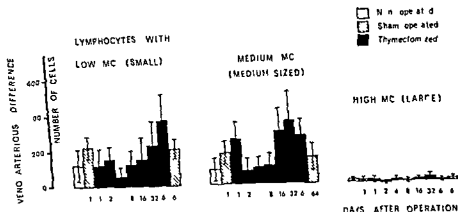


Fig 3

Difference between number of lymphocytes per mm³ of blood from a splenic vein and a splenic artery at different intervals after thymectomy and sham operation The lymphocytes are subdivided into cells with low medium and high mitochondrial content (MC) Mean ± S E

son 1967) The second increase seems to be an increase with age (*cf* *Ernstrom & Larsson* 1966)

Thymectomized Animals

Lymphocytes as well as granulocytes were increased in number 1 and 2 days after thymectomy in comparison with the values in the non-operated animals After 4 days the number was about normal again Later after operation (16 32 and 64 days) the number of lymphocytes (Fig 2) and granulocytes was increasing As no significant differences between the values in sham operated and thymectomized animals were found the changes cannot be ascribed to a specific effect of thymectomy (Table 1)

The subdivision of the lymphocyte population showed that the changes in total lymphocyte number after thymectomy were reflected in all three main subpopulations of lymphocytes although they were most pronounced in the medium sized lymphocytes with medium mitochondrial content (Fig 2)

The splenic veno arterial difference in total number of lymphocytes was slightly increased 1 day after thymectomy but not to a significant level and did not differ from the increase in sham thymectomized animals The difference decreased in the period from 1 to 4 days From 4 to 64 days after operation the splenic veno arterial differences increased successively (Fig 1)

The subdivision of the lymphocyte population showed that early after thymectomy (1 8 days) the splenic veno arterious difference in number of lymphocytes of the smallest type with 0-5 mitochondria per cell was small in comparison to that in the non operated and sham

TABLE 1

Number of Lymphocytes and Granulocytes in Splenic Venous and Arterial Blood of Normal Guinea Pigs and of Thymectomized and Sham Operated Guinea Pigs 1 and 64 days after Operation

	Interval after operation	Number of lymphocytes per mm³								Number of granulocytes	
		Total no.		No. with low MC		No. with medium MC		No. with high MC			
		SV	SA	SV	SA	SV	SA	SV	SA	SV	SA
Thymectomized	1	1155 ±201	1[illegible]78 ±110	7[illegible]3 ±88	633 ±69	872 ±119	624 ±51	30 ±7	21 ±[illegible]	1817 +264	1306 ±147
Sham operated	1	1499 ±133	108[illegible] ±107	683 ±76	401 ±[illegible]3	763 ±60	594 ±51	36 ±5	30 ±4	1441 ±140	1298 ±174
Thymectomized	64	2420 ±213	1[illegible]39 ±2[illegible]1	1139 +1[illegible]8	7[illegible]5 ±96	1237 +110	987 ±1[illegible]	44 ±7	37 ±5	1956 ±187	1690 +240
Sham operated	64	2195 ±190	1857 ±197	998 +79	793 ±77	1139 ±112	1016 ±17	58 ±10	46 ±8	1963 ±193	1691 +162
Non-operated		1309 ±118	1098 +99	71[illegible] +67	593 +57	572 +48	490 ±44	24 +3	14 +4	1048 ±95	1043 ±133

Mean no. of cells per mm³ of blood ± SE. SV = Splenic vein. SA = Splenic artery. No significant difference between corresponding thymectomized and sham operated animals was demonstrated.

TABLE 2

Effect of Thymectomy on the Splenic Veno Arterious Difference in Number of Lymphocytes Belonging to the Class Characterized by 0–5 Mitochondria per Cell (Smallest Lymphocytes)

	Days after operation						
	1	2	4	8	16	32	64
Thymectomized	7±19	7±12	8±21	—4±8	24±13	23±37	64±28
Sham operated	32±14						44±21
Non operated	29±19						

No per mm³ Mean Difference ± S E
Denotes significant export p < 0 05

operated (Table 2) A similar low output of small and medium sized lymphocytes was found 2–8 days after thymectomy Later after thymectomy the increasing veno arterious difference in total number of lymphocytes was due to an increasing difference in number of small and medium sized cells with low and medium mitochondrial content (Fig 3)

DISCUSSION

In a previous paper a splenic veno arterious difference of lymphocytes was reported (*Ernstrom & Sandberg* 1968) The present investigation has shown that the excess lymphocytes in the splenic veins compared to the artery belong to all categories of lymphocytes characterized by different mitochondrial content and different size This is in contrast to the export of lymphocytes from the thymus consisting exclusively of small cells with few mitochondria in their cytoplasm (*Ernstrom* 1965)

Thymectomy resulted in an increased number of lymphocytes and granulocytes in the blood after 1 and 2 days This was not however a specific effect of thymectomy as it was also observed in the sham thymectomized animals At 4 days the number of white blood cells was normal again At longer intervals after thymectomy the number of lymphocytes was successively increasing This seems to be the normal increase in number of small and medium sized lymphocytes occurring in growing guinea pigs (*Ernstrom & Larsson* 1966)

The splenic veno arterious difference in number of lymphocytes was slightly increased 1 and 2 days after thymectomy and decreased to normal again at 4 days As regards the smallest lymphocytes with 0–5 mitochondria per cell the output was diminished to subnormal level 1 8 days after thymectomy This decrease may be compared to a similar but more pronounced decreased output of splenic lymphocytes after combined thymectomy and steroid treatment (*Ernstrom & Sandberg* 1969)

At later intervals up to 64 days after thymectomy a successively in

creasing output was observed. This increase consisting of small and medium sized lymphocytes may be a late effect of thymectomy but may also be an increase dependant on age. The relation between the splenic output of lymphocytes and the age of the guinea pig will be further studied in a following investigation.

SUMMARY

The lymphocyte population of blood from a splenic vein and a splenic artery was studied in normal intact guinea pigs, in thymectomized and sham thymectomized guinea pigs. The investigation was performed at intervals of 1 to 64 days after operation. The number of lymphocytes and granulocytes per mm³ of blood was determined. The lymphocyte population was divided into subgroups classified by their mitochondrial content, which is correlated to the size of the lymphocyte. Special attention was focused on the difference between lymphocyte number in the blood from the splenic vein and the splenic artery, indicating migration of cells to or from the spleen. The following observations were made:

1. The exported lymphocytes from the spleen of normal young guinea pigs belong to all categories of lymphocytes characterized by different mitochondrial content.

2. Increased output of splenic blood lymphocytes was observed 1 day after thymectomy, followed by decrease to or below normal levels at 2–8 days, after which a successive increase was found to the end of the experiment.

3. An early increase in the output of lymphocytes was observed after sham operation as well as after thymectomy, i.e. the increased export of white blood cells from the spleen shortly after thymectomy is most probably a non specific operative effect.

4. The number of lymphocytes in the blood varied in a similar way as the splenic export of lymphocytes. The increased number and output at later periods of the experiments may depend on increasing age of the experimental animals.

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REGULATION OF OUTPUT OF LYMPHOCYTES FROM THE SPLEEN

2 A Quantitative Investigation in Sham Operated and Thymectomized Guinea Pigs During Steroid Induced Involution and Regeneration

By

ULF ERNSTROM and GORAN SANDBERG

Received 2 xii 68

Steroid hormones take part in the regulation of the growth and mass of the thymo lymphatic tissue Thus adrenalectomy and castration cause lymphatic hyperplasia while excess of steroids causes lymphatic atrophy (for ref see *Dougherty* 1952) The steroid induced lymphatic involution is most pronounced in the thymic cortex Nuclear pyknosis and cellular lysis occur within the first hours after administration of steroids Inhibition of the synthesis of proteins and nucleoproteins by the thymolytic steroids has also been reported (for ref see *Ernstrom & Larsson* 1967) After a delay of some days the steroids cause a marked reduction in the export of thymic lymphocytes at least in guinea pigs (*Ernstrom & Larsson* 1967)

The first question to be investigated in the present paper was the possible influence of steroids on the output of lymphocytes from the spleen either early during the phase of involution or late during the phase of lymphatic regeneration

In several rodents the thymus is necessary for normal growth and differentiation of the non thymic lymphatic tissue during early post natal stages (*Arsenberg et al* 1962 *Arnason et al* 1962 *Martinez et al* 1962 *Miller* 1962 *Sherman et al* 1963) In animals more mature at birth (e g the guinea pig) and in older animals thymectomy has less or no significant influence on the mass or histological appearance of the lymphatic organs In agreement herewith no conspicuous influence of thymectomy on the output of lymphocytes from the normal spleen in guinea pigs could be demonstrated (*Ernstrom et al* 1969)

Even in mature animals however quantitative studies have disclosed a role of the thymus in the regeneration of lymphatic tissue after involution caused by X irradiation (*Globerson et al* 1962 *Miller* 1962

The expenses of this investigation were defrayed by a grant from the Swedish Cancer Society

Miller et al 1963 *Cross et al* 1964 *Globerson & Feldman* 1964) In a previous paper an influence of the thymus on the regeneration of the spleen after steroid induced involution was demonstrated in guinea pigs—regeneration being retarded in thymectomized animals (*Ernstrom & Gyllensten* 1965)

The second question investigated in the present paper was the possible influence of the thymus on the output of lymphocytes from the spleen during steroid induced involution and regeneration

MATERIAL AND METHODS

Totally 200 male guinea pigs with an initial weight of 242 ± 3 g (mean ± S.E.) were used The animals were fed on cabbage turnips carrots and vitamin pellets They were divided into two main groups

Sham operated guinea pigs injected with prednisolone (98 animals)

Thymectomized guinea pigs injected with prednisolone (102 animals)

Thymectomy and sham operation were performed under local anesthesia (subcutaneous infiltration of 0 5 per cent Xylocaine® Astra) according to the technique of *Gyllensten* (1953)

Prednisolone (Ultracortenol® Ciba) was administered i p in a single *dose* of 50 mg/kg b w The injection was given immediately after operation

The animals were investigated 1 3 and 6 hours and 1 3 6 and 9 days *after* operation and steroid treatment On investigation the guinea pigs were anaesthetized with 2 5 per cent Nembutal sodium (25–50 mg/kg b w) Blood samples were taken from a splenic vein and a splenic artery (for details see the previous paper in this series)

The blood samples were used for white cell counts in a Bürker counting chamber with differentiation between polynuclear and mononuclear cells and for differentiation of lymphocytes into subclasses (including cells with *different* mitochondrial content) in preparations stained supravitally with Janus green B and neutral red (for details see Ernström et al 1969)

Lymphocytes with 0 10 mitochondria are denoted as cells with low mitochondrial content (low MC) lymphocytes with 11–20 mitochondria as cells with medium mitochondrial content (medium MC) and lymphocytes with >20 mitochondria as cells with high mitochondrial content (high MC)

The number of lymphocytes and granulocytes per mm³ of blood from the splenic artery and the splenic vein was calculated The splenic veno arterial differences in the number of different white blood cells were obtained from each animal and all differences were then analysed statistically by Student s *t* test

RESULTS

Sham Operated Animals Treated with Steroid

The total number of lymphocytes was only slightly depressed by steroid treatment Minimal values were recorded at 6 hours and 6 days after steroid administration with a transient increase to the normal level at 1 day

A subdivision of the lymphocyte population showed that a biphasic change in the lymphocyte number in the blood after steroid injection occurred in all subpopulations although most markedly in the lymphocytes with low and medium mitochondrial content (Fig 1)

The splenic veno arterial difference in number of lymphocytes was not depressed by steroid treatment—normal difference being about 200 cells per mm³ (*Ernstrom et al* 1969) On the contrary a high

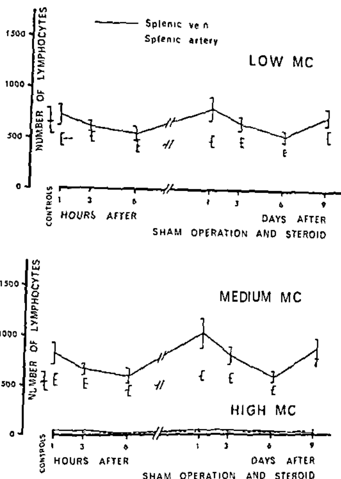


Fig 1

Number of lymphocytes per mm³ of blood from a splenic vein and a splenic arter at different intervals after sham operation and treatment with prednisolone The lymphocytes are subdivided into cells with low medium and high mitochondrial content (MC) Mean ± SE

export value was registered 1 day after steroid the difference being 772 ± 254 cells (Fig 2) A subdivision of the lymphocytes showed that all categories of lymphocytes were exported in large number at this time (Fig 3) A significant export of small lymphocytes with low mitochondrial content was found at all time intervals after treatment (Fig 3) A significant export of medium sized lymphocytes with medium mitochondrial content also occurred at all times except 9 days after treatment (Fig 3)

Pronounced granulocytosis occurred 3 and 6 hours after steroid administration At 24 hours the number of granulocytes *in the blood* was normal again (Fig 4a) Apart from this early granulocytosis no change in blood granulocytes was recorded

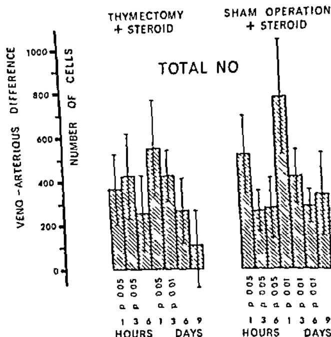


Fig 2

Difference between number of lymphocytes per mm³ of blood from a splenic vein and a splenic artery at different intervals after sham operation or thymectomy and treatment with prednisolone Mean ± S E

Thymectomized Animals Treated with Steroid

No significant depression of the total lymphocyte count occurred As in the sham operated animals a high lymphocyte number was noted after 1 day and lower values after 3 6 and 9 days A subdivision of the lymphocyte population demonstrated that both lymphocytes with low and medium mitochondrial content followed the same course (Fig 5)

The splenic veno arterial difference in number of lymphocytes was high 1 day after steroid injection and operation as in the sham operated animals This difference subsequently decreased successively to a minimal value of 109 ± 192 cells per mm³ after 9 days (no significant export Fig 2) The subdivision of the lymphocyte population demonstrated that the lymphocytes with low and medium mitochondrial content behaved identically i e, a large difference was present after 1 day followed by a decreasing difference to a minimal value after 9 days (Fig 3) As regards the smallest type of lymphocytes with 0 5 mitochondria per cell the splenic output was lower in the thymectomized than in the sham-operated animals at all corresponding intervals after steroid treatment This deficient export in the thymectomized steroid treated guinea pigs was most pronounced after 9 days (Table 1)

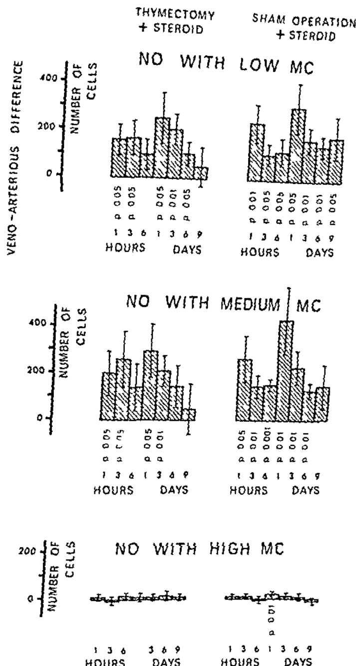


Fig 3

Difference between number of lymphocytes per mm³ of blood fr m a splenic vein and a splenic artery at different intervals after sham operation r thymectomy and treatment with prednisolone The lymphocytes are subdivided int cells with low medium and high mitochondrial content (MC) Mean ± SE

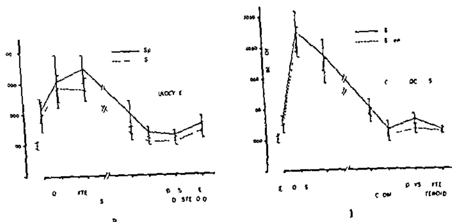


Fig. 4

Number of granulocytes per mm³ of blood from a splenic vein and a splenic artery at different intervals after sham operation (a) or thymectomy (b) and treatment with prednisolone. Mean ± S.E.

TABLE 1

Splenic Veno-Arterious Difference in Number of Lymphocytes Belonging to the Class Characterized by 0–5 Mitochondria per Cell (Smallest Type of Lymphocytes)

	Interval from operation and treatment						
	1 h	3 h	6 h	1 day	3 days	6 days	9 days
Thymectomized steroid treated	22±12	12±11	14±10	23±18	9±11	8±15	0±18
Sham operated, steroid treated	28±12	17±9	15±7	40±16	22±8	10±10	28±10

Lower values in thymectomized than in sham operated animals. No. per mm³. Mean difference ± S.E. denotes significant export $p < 0.05$

The granulocytes increased in number from the normal values of around 1000 cells per mm³ to about 4000 cells at 3 and 6 hours after thymectomy and steroid administration (Fig. 4b).

DISCUSSION

In guinea pigs with intact thymus, steroid treatment caused no depression of the splenic export of lymphocytes. This is in contrast to the decreased export from the thymus (*Ernstrom & Larsson* 1967). The difference in response to steroids indicates the existence of biological differences between thymic and splenic lymphocytes.

The present investigation as well as a previous one (*Ernstrom & Larsson* 1967) disclosed a large number of circulating blood lymphocytes 24 hours after steroid treatment irrespective of thymectomy. Concurrently a large export of lymphocytes from the spleen was

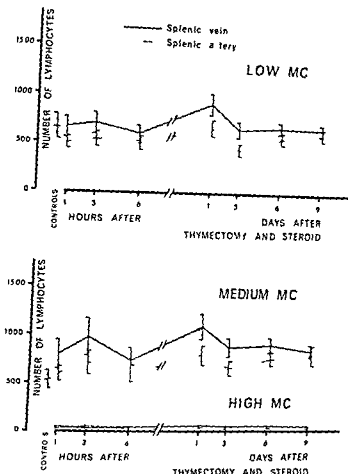


Fig 5

Number of lymphocytes per mm³ of blood from a splenic vein and a splenic artery at different intervals after thymectomy and treatment with prednisolone. The lymphocytes are subdivided into cells with low, medium and high mitochondrial content (MC). Mean ± SE

found the export including all categories of lymphocytes. It is not known whether the emigrating splenic cells are important for the regeneration of other lymphatic tissues damaged by the steroids.

In the thymectomized guinea pigs treated with steroid the splenic export of lymphocytes decreased during the phase of lymphatic regeneration (3, 6 and 9 days after steroid – *Gyllensten* 1962). At 9 days after steroid administration the total export was only 30 per cent of that in the sham operated animals and no export of the smallest type of lymphocytes occurred. This decrease was much more pronounced than that after thymectomy without steroid (*Ernström et al* 1969).

Thus thymectomy interferes with the export of lymphocytes from the spleen during lymphatic regeneration after steroid induced involution. This finding may be regarded as an analogue to the retarded

regeneration of steroid involuted lymphatic tissue in thymectomized guinea pigs (*Ernstrom & Gyllensten* 1965) and to the delayed immunological restitution after irradiation of the lymphatic tissue in thymectomized adult mice (*Globerson et al* 1962 *Miller et al* 1963)

The result may be explained by a migratory stream of lymphocytes from the thymus to the regenerating spleen this flow of cells being necessary for normal splenic regeneration and export of lymphocytes However the additional influence of a thymic hormone on the spleen cannot be excluded

SUMMARY

The lymphocyte populations of blood from a splenic vein and a splenic artery were studied in sham operated and thymectomized guinea pigs during steroid induced involution and regeneration of the lymphatic tissue Special attention was focused on the splenic veno arterial difference in lymphocyte number indicating an export or import of lymphocytes in the spleen The following observations were made

1 At 24 hours after steroid administration the blood contained a large number of lymphocytes irrespective of thymectomy Concurrently a large export of splenic lymphocytes occurred also irrespective of thymectomy

2 In sham operated guinea pigs no depression of the splenic export of lymphocytes was noted after steroid treatment

3 In the thymectomized guinea pigs the splenic export of lymphocytes decreased during lymphatic regeneration after steroid induced involution This was most pronounced in the smallest lymphocytes A migration of small lymphocytes from the thymus to the spleen may be necessary for normal splenic regeneration

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HISTOLOGICAL STAINING PROPERTIES OF *IN VITRO* FORMED FIBRIN CLOTS AND PRECIPITATED FIBRINOGEN

By

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Received [illegible]

Common techniques for the demonstration of fibrin in histological sections are immunofluorescence technique and various histological staining methods among which Mallory's phosphotungstic acid haematoxylin (PTAH) (19) and the trichrome methods with modifications (14) are most commonly used

The immunofluorescence technique is believed to be the one most specific for the demonstration of fibrin (5 13) The PTAH and the trichrome methods seem to be more unspecific although these methods are used in histological routine work more than the others

The mechanism of the staining reactions obtained by using the PTAH and the trichrome methods is largely unknown (2 14) Occasionally the demonstration of fibrin by the PTAH or trichrome methods seems to fail Thus if fibrin clots are formed by purified fibrinogen and thrombin alone a negative result is obtained with the PTAH (8) and Lendrum's Martius scarlet blue (MSB) (10) methods This may be related to the lack of fibrin stabilizing factor (FSF) which is suggested to promote the formation of disulphide bonds resisting the action of solvents such as 30 per cent urea or 1 per cent monochloracetic acid (16) However electron microscopical examinations of fibrin prepared from normal plasma and plasma from a patient with FSF deficiency showed no structural difference between the two types of fibrin when stained with phosphotungstic acid (6 12)

The characteristics of the fibrin clot are probably influenced by several factors other than FSF Thus fibrin aggregation is enhanced by calcium (9) as well as by colloids (1)

Fibrinogen the precursor of fibrin is easily precipitated *in vitro* without actual transformation into fibrin (7 9) To what extent

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precipitation occurs *in vivo* is unknown but it may be a potential source of error in histological staining of fibrin

The aim of this study is to examine under which conditions fibrin fibrinogen and globulins treated by histological technique give the specific staining reactions when the PTAH method the trichrome methods and the immunohistochemical technique are used

MATERIALS

Fibrinogen Purified human fibrinogen (Kabi Stockholm Sweden) about 95 per cent clottable was used It was dissolved in 0 3 M NaCl adjusted to pH 7 4 with 1 N NaOH and dialysed against 0 3 M NaCl buffered with 1/10 volume Owren s buffer for 4 hours After the dialysis the concentration of fibrinogen was 1 2 per cent

Citrated platelet poor plasma Nine volumes of blood from healthy fasting subjects were collected into bottles containing one volume of 3 1? per cent sodium citrate solution After centrifugation for 60 minutes at 2000 g the plasma was pipetted off recentrifuged and stored at −20 C.

Plasma with defect FSF (Fibrin Stabilizing Factor) function Platelet poor citrated plasma was obtained from a 24 years old female patient (kindly supplied by Dr H C Godal) She had a moderate bleeding tendency (haemorrhages after partus and after tooth extraction) Laboratory examination revealed no other coagulation defect than a deficient FSF function Further studies disclosed the presence of a strong inhibitor of FSF in the patient s plasma.

Serum Whole blood from normal subjects were collected in a glass bottle and incubated at 37 C for one hour After removal of the clot by centrifugation for 30 minutes serum was incubated at 37 C overnight. The stored serum did not clot fibrinogen

Serum fibrinogen mixture Fibrinogen was dialysed against a 3 1? per cent sodium citrate solution for 24 hours at ? C. One volume of this solution was mixed with 4 volumes of normal serum

Macroglobulin serum was obtained from a 80 years old male patient with macroglobulinaemia Waldenström The patient presented moderate anaemia typical sternal marrow findings and a moderate bleeding tendency The serum contained about 3 per cent of γM globulin

Purified macroglobulin One volume of serum from the patient with macroglobulinaemia was diluted with 9 volumes of distilled water After centrifugation the supernatant was discarded The precipitate was dissolved in 0 3 M NaCl and reprecipitated twice

Owren s buffer A modified veronal buffer pH 7 38 ionic strength 0 15 (20)

Thrombin Highly purified human thrombin was prepared by the method of Berg et al (3) and contained 30 NIH units per ml in distilled water

Human serum albumin A 20 per cent aqueous solution of human serum albumin was obtained from Kabi Stockholm Sweden This solution was diluted with distilled water to a final concentration of 4 per cent

Dextran A 10 per cent solution of Dextran 40 Rheomacrodex in 0 15 M NaCl (Pharmacia Uppsala Sweden) Dilutions were performed with 0 15 M NaCl

Glycine methyl ester HCl (Sigma Chemical Company St Louis Missouri U S A) An 0 1 M solution was adjusted to pH 7 4 Glycine methyl ester is an inhibitor of fibrin cross linking (18)

Antisera The following commercial antisera (Hyland Laboratories Los Angeles California U S A) were used

rabbit anti human fibrin labelled with fluorescein isothiocyanate FITC)
rabbit anti human fibrinogen labelled with FITC
rabbit anti human albumin labelled with FITC
goat anti rabbit γ globulin labelled with FITC

By immunoelectrophoresis against normal human plasma the antiserum against fibrin showed one precipitation line in the β region Anti fibrinogen serum showed a distinct line in the same position and a very faint line probably corresponding to α macroglobulin Anti albumin showed only one precipitation line

By gel diffusion tests anti fibrinogen serum showed one precipitation line against plasma diluted 1 2 and 1 10 and *no* line against normal human serum diluted 1 2 and 1 10 Anti albumin serum showed one precipitation line against human serum albumin (1 mg/ml) and normal human serum diluted 1 5 and 1 20

METHODS

Clots

The clots were prepared in lustroid tubes Unless otherwise stated the tubes were immersed in a waterbath at 37 C for three hours prior to the addition of fixation fluid

Fibrin clots prepared from purified fibrinogen The fibrinogen solution (0 2 ml) was mixed with one of the following test solutions at 37 C

(1) 0 2 ml of Owren s buffer + 0 2 ml of H_2O
(2) 0 2 ml of 50 mM $CaCl_2$ + 0.2 ml of H_2O
(3) 0 2 ml of 50 mM $CaCl_2$ + 0 2 ml of glycine methyl ester
(4) 0.2 ml of albumin + 0 2 ml of Owren s buffer

Thereafter 0 2 ml of thrombin was added One sample with each test solution was left undisturbed after addition of thrombin Clotting took place within 10 seconds Another sample was stirred with a glass or wooden rod during clotting after addition of thrombin

Other samples with test solution (1) and thrombin were incubated for three hours Thereafter ultracentrifugation was carried out (Spinco L 2 rotor 40) at 35 000 rpm (111 000 g at tip) for three hours at 35 –37 C or incubated at 2 C for three hours prior to fixation

Fibrin clots prepared from citrated plasma Citrated plasma (0 2 ml normal or with defect FSF function) was mixed with

(1) 0 4 ml of Owren s buffer
(2) 0 2 ml of 50 mM $CaCl_2$ + 0 2 ml of Owren s buffer

and 0 2 ml of thrombin was added One sample with either test solution was left undisturbed another sample was stirred Samples with test solution (1) were also subjected to ultracentrifugation as described above

Fibrin clots prepared from mixtures of serum and fibrinogen These were made and treated as clots from citrated plasma

Whole blood clots Samples of one ml of whole blood from normal subjects were collected without anticoagulant Without stirring clotting took place in about one hour In the samples which were stirred with a glass rod clotting took place in about 30 minutes

Solubility The clots formed by addition of thrombin to purified fibrinogen were soluble in 30 per cent urea or one per cent monochloracetic acid within 3 hours and insoluble when formed in the presence of $CaCl_2$

Clots formed by addition of thrombin to purified fibrinogen in the presence of $CaCl_2$ and glycine methyl ester were soluble in urea or monochloracetic acid within 3 hours thus demonstrating inhibition of FSF Likewise clots formed by addition of thrombin to citrated plasma with defect FSF function in the presence of $CaCl_2$ were soluble in urea or monochloracetic acid within 3 hours

Precipitates

Precipitates were prepared in lustroid tubes at 2 C After incubation the tubes were centrifuged at 7000 g for 30 minutes The supernatant was removed and fixation fluid added to the precipitates

Purified fibrinogen Fibrinogen solution (0 2 ml) was diluted with

(1) 5 ml of 0 014 per cent acetic acid
(2) 0 4 ml of 50 mM $CaCl_2$ + 2 5 ml H_2O
(3) ml of 1 5 per cent dextran

Incubation time was 3 hours

Citrated plasma

(1) 0 2 ml of citrated plasma + 4 5 ml of 0 014 per cent acetic acid
(2) 1 ml of citrated plasma + 0 5 ml of 10 per cent dextran

Incubation time was 18 hours

Serum fibrinogen mixture One ml of serum fibrinogen mixture was diluted with 0 5 ml of 10 per cent dextran Incubation time was 18 hours

Euglobulin fractions Normal serum (0 5 ml) serum from a patient with macroglobulinaemia Waldenstrom (0 5 ml) or purified macroglobulin solution (0 5 ml) was diluted with

(1) 9 5 ml of 0 014 per cent acetic acid
(2) 0 1 ml of 3 12 per cent sodium citrate + 9 5 ml of 0 014 per cent acetic acid
Incubation time was 10 minutes

Preparation and Staining of Sections

Studies using conventional histological technique All clots and precipitates were fixed in Zenker s fluid for 24 hours and rinsed in flowing tap water for another 24 hours or fixed in 8 per cent formaldehyde with 5 per cent mercuric chloride for 3 days

Specimens fixed in Zenker s fluid were stained with Mallory s phosphotungstic acid haematoxylin (PTAH) (19) Specimens fixed in formaldehyde/mercuric chloride were stained with Heidenhain s azan stain (29) Lendrum s Martius scarlet blue method and Masson 44/41 method (14)

In the following red stain using MSB and Heidenhain s azan methods dark blue using Masson 44/41 and bluish black using PTAH will be designated as + (positive) Blue stain using MSB Heidenhain s azan and Masson 44/41 methods as well as red brownish stain using PTAH method will be designated as – (negative) Where intermediate staining occurred the symbol +– will be used In some sections the material mostly gave a positive stain but in smaller areas the stain was negative or vice versa These instances will be marked +(–) or –(+) respectively

Immunofluorescence studies The following specimens were used

(1) fibrinogen precipitated from citrated plasma with acetic acid
(2) euglobulin fractions from serum
(3) euglobulin fractions from macroglobulin serum
(4) purified macroglobulin
(5) fibrin clots prepared from purified fibrinogen
(6) as (5) with addition of albumin
(7) fibrin clots prepared from citrated plasma
(8) as (7) with CaCl

The precipitates were placed in a slit in a piece of liver from mouse

The specimens were frozen by CO_2 gas sectioned immediately at 6–8 micron in a Tissue Tek Cryostat (Ames Lab Tek) and stained the same day using the direct method of *Coons & Kaplan* (4) The sera were applied undiluted and diluted 1 10 with 0 3 M NaCl

Fluorescence microscopy was performed with a Leitz Orthomat microscope using UG 1 and BG 38 filtres Photographs were taken with Agfa Isopan IFF film (15 din)

Controls

(1) Unstained sections showed scarcely visible autofluorescence
(2) There was no binding of goat anti rabbit γ globulin labelled with FITC
(3) Anti fibrin and anti fibrinogen labelled with FITC were adsorbed with equal amounts 0 6 per cent purified fibrinogen Anti albumin labelled with FITC was adsorbed with equal amounts of 1 per cent human serum albumin By gel diffusion tests anti fibrinogen labelled with FITC adsorbed with fibrinogen showed a precipitation line against anti fibrinogen labelled with FITC and no line against normal human plasma diluted 1 1 and 1 10 Anti albumin labelled with FITC adsorbed with albumin showed one precipitation line against anti albumin labelled with FITC The anti fibrinogen and anti albumin sera are therefore adsorbed in excess Sections treated with adsorbed sera showed no fluorescence except for the autofluorescence

RESULTS

Studies Using Conventional Histological Technique

Clots

The results of staining reactions of fibrin clots are shown in Tables 1 2 and 3

Clots from normal plasma plasma with defect FSF function and serum fibrinogen mixture stained identical

Stirring with wooden rods and stirring with glass rods gave identical staining results

TABLE 1

Fibrin Clots Formed from Purified Fibrinogen and Thrombin

	PTAH	MSB Masson 44/41 Heidenhain s azan
Purified fibrinogen	—	—
Purified fibrinogen with		
Stirring	+ (—)	+
Ultracentrifugation	+	—
Cooling	+ (—)	+
CaCl	+	+
CaCl + Stirring	+	+
CaCl + Glycine methyl ester	+	+
Albumin	+	—
Albumin + Stirring	+	+

TABLE 2

Fibrin Clots Formed from Citrated Plasma and Thrombin

	PTAH	MSB Masson 44/41 Heidenhain s azan
Citrated plasma	+	—
Citrated plasma with		
Stirring	+	+
Ultracentrifugation	+	—
CaCl	+	—
CaCl + Stirring	+	+

Clot prepared from plasma with defect FSF function behaved like those from normal plasma.

TABLE 3

Clots Prepared from Whole Blood

	PTAH	MSB Masson 44/41 Heidenhain s azan
Whole blood	+	—
Whole blood + Stirred	+	+


2
3
4
5
7
8

PTAH All fibrin clots derived from fibrinogen or normal plasma and formed by the addition of $CaCl_2$ revealed coarse threads (Fig. 1) whereas the lack of calcium or fibrin stabilizing factor (FSF) tended to result in more delicate fibrin strands (Figs 2–5)

When stirring was performed during clotting the clots were composed of twisted bundles of fibres of varying thickness (Fig. 6) Clots prepared by purified fibrinogen and thrombin and kept at 2° C before fixation had a similar appearance (Fig. 7)

Ultracentrifugation after the clot was formed gave a central compact mass whereas delicate fibrillar structures could be discerned in the periphery (Fig. 8)

A positive stain for fibrin was dependent on the addition of $CaCl_2$ or the presence of serum colloids (whole serum or purified albumin) Thus clots formed by purified fibrinogen and thrombin gave a negative stain (Fig. 2) whereas all other clots gave a positive reaction (Figs 1 and 3–8)

MSB Masson 44/41 Heidenhain's azan In fibrin clots the thickness of the individual strands varied less than the thickness of strands stained with PTAH However clots formed by purified fibrinogen and thrombin and by purified fibrinogen and thrombin with the addition of glycine methyl ester and $CaCl_2$ had definitely finer strands than the others

Stirring during clotting, cooling or ultracentrifugation of the clot revealed a microscopical appearance similar to that described for PTAH stained clots

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Figs 1–8

Fig. 1 Fibrin clot formed by citrated plasma and thrombin with the addition of $CaCl_2$ The strands are coarse PTAH gives a positive stain PTAH × 1200

Fig. 2 Fibrin clot formed by purified fibrinogen and thrombin The strands are very fine PTAH gives a negative stain PTAH × 1200

Fig. 3 Fibrin clot formed by purified fibrinogen and thrombin with the addition of $CaCl_2$ and glycine methyl ester for inhibition of FSF-contaminants The strands are very fine PTAH gives a positive stain PTAH × 1200

Fig. 4 Fibrin clot formed by purified fibrinogen and thrombin with the addition of albumin The strands are fine PTAH gives a positive stain PTAH × 1200

Fig. 5 Fibrin clot formed by citrated plasma from a patient with defect FSF function and thrombin with the addition of $CaCl_2$ The strands are fine PTAH gives a positive stain PTAH × 1200

Fig. 6 Fibrin clot formed by purified fibrinogen and thrombin The sample was constantly stirred with a glass rod during clotting The clot is composed of more or less densely packed and twisted bundles of fibrin strands PTAH gives a positive stain PTAH × 1200

Fig. 7 Fibrin clot formed by purified fibrinogen and thrombin The sample was cooled before fixation The fibrin strands are packed in coarse bundles PTAH gives a positive stain PTAH × 1200

Fig. 8 Fibrin clot formed by purified fibrinogen and thrombin Ultracentrifugation was carried out after the clot was formed The clot has a compact centre whereas delicate strands may be discerned at the periphery PTAH gives a positive stain PTAH × 1200

In all sections the MSB Masson 44/41 and Heidenhain's azan stains gave corresponding results

A positive stain was dependent on the addition of CaCl or on physical alterations (stirring or cooling) of the clot The presence of serum colloids (whole serum or purified albumin) seemed to prevent fibrin from taking the positive stain even when CaCl was added However, a positive stain was always attained when stirring was carried out during clotting

Likewise whole blood gave a negative reaction (Table 3) If stirring was performed during clotting the reaction was again reversed

Precipitates

Fibrinogen precipitated with dextran showed traces of fibrillary structure The other precipitates were composed of granular homogeneous masses In the presence of CaCl the granules appeared more coarse

Except for two specimens (see Tables 4 and 5) the MSB Masson 44/41 and Heidenhain's azan gave corresponding results

Fibrinogen precipitates (Table 4) Purified fibrinogen precipitated by dilution with acetic acid or CaCl gave an overall positive staining result by all methods investigated

When purified fibrinogen was precipitated in the presence of dextran an intermediate staining reaction occurred

When serum colloids were present PTAH still gave a positive stain whereas the other methods were negative

Serum precipitates (Table 5) Euglobulin from normal serum showed intermediate staining reactions with PTAH and negative reactions with the other methods

Precipitated macroglobulin gave a positive stain with MSB Masson 44/41 and Heidenhain's azan whereas PTAH was negative

TABLE 4

Fibrinogen Precipitates

	PTAH	MSB Masson 44/41 Heidenhain's azan
Purified fibrinogen with		
Acetic acid	+ (−)	+ (—)
CaCl	+	+
Dextran	+ −	+ —
Citrated plasma with		
Acetic acid	+ (—)	+ −
Dextran	+	
Serum fibrinogen mixture with		
Dextran	+	—

MSB was negative Masson 44/41 and Heidenhain's azan were positive

TABLE 5

Serum Precipitates

	PTAH	MSB Masson 44/41 Heidenhain's azan
Euglobulin from normal serum	+ —	—
Euglobulin from macroglobulin serum	—	+
Purified macroglobulin	—	+

Identical reactions were obtained with citrated serums, except for MSB was positive Masson 44/41 and Heidenhain's azan were negative

TABLE 6

Immunofluorescence Studies

	anti fibrin	anti fibrinogen	anti albumin
Clots			
Purified fibrinogen			
Thrombin	+	+	—
Purified fibrinogen			
Albumin – Thrombin	+	+	(+)
Citrated plasma			
Thrombin	+	+	+
Citrated plasma			
CaCl Thrombin	+	+	+
Precipitates			
Citrated plasma			
Acetic acid	+	+	+
Euglobulin from serum	—	—	(+)
Euglobulin from			
macroglobulin serum	—	—	(+)
Purified macroglobulin	—	—	—

(+) is indicated where weak fluorescence was obtained

Immunofluorescence Studies (Table 6)

Fibrin clots and fibrinogen precipitates showed specific fluorescence for both fibrin and fibrinogen (Figs 9 and 10)

Fibrinogen precipitated from citrated plasma showed binding of anti albumin labelled with FITC and mostly at the surface (Fig 11) Similar results were obtained in fibrin clots formed from citrated plasma by thrombin

Euglobulin fractions precipitated from serum and macroglobulin serum showed a weak fluorescence for albumin Precipitates from purified macroglobulin showed a minimal fluorescence for albumin which could not be reproduced in photographs

Serum euglobulin fractions and purified macroglobulin showed no binding of anti fibrin or anti fibrinogen labelled with FITC

There was no binding of antisera to mouse liver



Figs 9–11

Fig 9 Fibrin clot formed by citrated plasma and thrombin Anti human fibrinogen labelled with FITC × 400

Fig 10 Fibrinogen precipitate from citrated plasma produced by acetic acid Anti human fibrin labelled with FITC × 400

Fig 11 Parallel section to that of Fig 10 Anti human albumin labelled with FITC × 400

DISCUSSION

In the present study a positive stain for fibrin was found to be dependent on the addition of CaCl no matter which method under investigation were used Furthermore a positive stain with PTAH was dependent on the presence of serum colloids whereas physical factors during clotting seemed to influence the stainability when the trichrome methods (MSB Masson 44 41 Heidenhain s azan) were used Whether or not the fibrin clot was urea soluble did not seem to affect the staining reactions

Both calcium and fibrin stabilizing factor (FSF) are required for the conversion of urea soluble fibrin to the urea insoluble type (15) When one of these substances is lacking or neutralized the fibrin remains urea soluble A reduction of the effect of FSF was attained by inhibition of the FSF contaminants in purified fibrinogen (17) by glycine methyl ester (18) or by use of plasma from a patient with a strong FSF inhibitor

Fibrin clots formed by purified fibrinogen and thrombin gave a negative stain whereas clots prepared from purified fibrinogen with added CaCl gave a positive stain whether or not glycine methyl ester was present Furthermore fibrin clots prepared by plasma from a patient with a FSF inhibitor stained like fibrin clots prepared by normal plasma Thus a positive stain for fibrin is dependent on CaCl but the presence of FSF activity does not seem to be a prerequisite for the positive reaction

However in histological sections the structure of the fibrin strands seems to be dependent on the urea solubility of the clot since all normal clots with the addition of CaCl showed coarse strands whereas the

lack of calcium of FSF tended to give finer strands *Duckert et al* (6) and *Lorand et al* (18) found no difference in the histological appearance of the fibrin clots prepared from plasma with calcium whether or not FSF was present but their experimental conditions differed from ours Since under the present experimental conditions FSF appears to be necessary for the formation of coarse fibrin strands our results may also indicate that the staining reactions are not definitely influenced by the calibre thickness of the individual strands

The presence of albumin in fibrin clots formed from purified fibrinogen resulted in a positive stain with PTAH This is in accordance with the results obtained by *Gitlin & Craig* (8) who got a positive stain with PTAH when albumin was present However these authors did not relate the positive reaction to the presence of albumin but to the urea insoluble form of fibrin an explanation which does not fit with our results

Purified fibrin clots with additional albumin and fibrin clots formed from plasma gave a negative stain with the trichrome methods even if $CaCl_2$ was added Therefore the presence of serum colloids during clotting seems to promote the positive PTAH stainability of fibrin whereas such substances apparently prevent fibrin from taking the positive stain with the other methods investigated

Aggregation of fibrin strands was produced by stirring or cooling during clotting or ultracentrifugation of the clot after it was formed All these procedures interfered with the staining reactions As to stirring or cooling the effect was particularly striking for the staining with the trichrome methods since this treatment of the clot always resulted in positive staining reactions independent of the clotting medium With constant stirring during clotting the mechanical distorsion of the clot produced alterations which cannot be related to increased aggregation of fibres only since ultracentrifugation which resulted in compression of the clots gave negative staining reactions with the three mentioned methods With regard to PTAH however fibre compression may play a role since clots formed during stirring or ultracentrifugation after the clot was formed both gave a positive stain

Like fibrin clots a positive stain of fibrinogen *in vitro* is apparently dependent on the medium in which the fibrinogen is precipitated Thus on the whole the same staining pattern as that of fibrin was obtained when $CaCl_2$ was added or serum colloids were present Thus in histological sections equivocal fibrin masses are presumably difficult to distinguish from precipitated fibrinogen when standard tinctorial techniques are used In this situation the immunofluorescence technique is not useful either since fibrin and fibrinogen are immunologically indistinguishable

Precipitated globulins gave a negative stain by PTAH whereas euglobulin from macroglobulin serum and purified macroglobulin

gave a positive stain with the trichrome methods This is in agreement with previous findings in biopsy materials from patients with macroglobulinaemia (11) Thus using the *trichrome* methods deposits of macroglobulins or substantial adsorption of macroglobulins to fibrin (21) may be mistaken for fibrin or fibrinogen

In conclusion, among the standard tinctorial methods investigated PTAH is found to be the one most specific for the demonstration of fibrin and fibrinogen *in vitro* but it fails when fibrin clots are formed by purified fibrinogen and thrombin only The trichrome methods give a positive stain of fibrin when the clot is changed by the influence of stirring during clotting or cooling Immunohistochemical technique seems to be the method of choice for the precise demonstration of fibrin or fibrinogen in histological sections However the latter substances cannot be distinguished by any of the methods used

SUMMARY

In order to test the specificity of the common histological stains for fibrin fibrin clots and precipitates of fibrinogen and globulins were prepared by histological technique and stained with Mallory s PTAH method or one of the trichrome methods (Lendrum s MSB and Masson 44/41 and Heidenhain s azan methods) Immunofluorescence technique was also applied to the clots and precipitates

All normal fibrin clots to which CaCl was added showed coarse strands in histological sections whereas the lack of calcium or fibrin stabilizing factor (FSF) tended to give more delicate fibrin strands

A positive stain for fibrin using the PTAH method was dependent on the addition of CaCl or the presence of serum colloids

A positive stain for fibrin using the trichrome methods was dependent on the addition of CaCl or physical alterations of the clot The presence of serum colloids seemed to prevent fibrin from taking the positive stain with the trichrome methods but a positive stain was always attained when stirring was carried out during clotting

FSF is probably not necessary for a positive stain for fibrin

Fibrinogen precipitates gave also positive stain with PTAH but again this was dependent on the addition of CaCl or the presence of serum colloids With the trichrome methods fibrinogen gave a positive reaction when CaCl was added whereas the presence of serum colloids gave on the whole negative results

Euglobulin fractions of macroglobulin serum and purified macroglobulin stained mainly as fibrin when the trichrome methods were used whereas PTAH was negative

Immunofluorescence technique applied to fibrin clots and fibrinogen precipitates revealed specific fluorescence for both fibrin and fibrinogen

It is concluded that among the standard tinctorial methods investi

lated PTAH is the one most specific for the demonstration of fibrin and fibrinogen in vitro

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DEPOSITS OF FIBRIN AND PLASMA PROTEINS IN THE NORMAL HUMAN PLACENTA

An Immunofluorescence Study

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In the normal human placenta eosinophilic extracellular deposits of fibrillar or granular appearance are seen in relation to the various elements of the trophoblast (Fig 1)

On the surface of the syncytium of the villi fibrin and fibrin like masses of various size are laid down (16) On the chorion plate similar deposits extend to finally form a continuous layer against the maternal blood space (Langhans layer) (16) Further masses of eosinophilic material are seen between the cells of the cell islands (14) and cyto trophoblastic cell columns (14) and of the cytotrophoblastic shell (13) In relation to the basal plate nearly continuous deposits are found both towards the intervillous space (Rohr s stria) and at the junctional zone between the cytotrophoblastic shell and the decidua (Nitabuch s layer) (13)

Many authors have studied these deposits the exact nature and origin of which have been much disputed (for review see *Moe* (13) *Moe* (14) and *Moe & Jorgensen* (16)) The major problem has been whether the deposits are composed of fibrin or other substances Previous studies have shown that Rohr s stria and the early deposits on the syncytium consist of maternal platelet thrombi and that later on increasing amounts of fibrin are added (13 16) In the advanced stages the deposits on the syncytium are homogeneous by light micro scopy Ultrastructurally they are composed of a finely granular material of moderate electron density (16) Because a few remaining platelets and scattered tracts of fibrillary fibrin were found in the granular masses it seems likely that the masses represent *old thrombi* (16) Granular masses with a similar electron microscopical appear

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The author is deeply indebted to *M Harbitz* M D *I Kornstad* M D and *H Orjasæter* M D for invaluable help

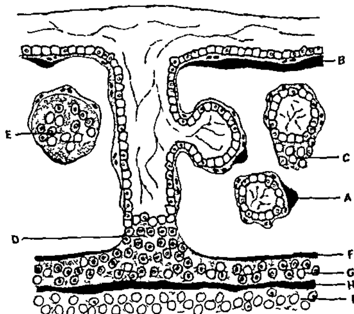


Fig 1

Deposits in the normal human placenta A) Deposits on the syncytium of the villi B) Langhans layer C) Intercellular deposits in the cytotrophoblastic cell columns D) Intercellular deposits in the anchoring villi F) Intercellular deposits in the cell islands F) Rohr s stria G) Intercellular deposits in the cytotrophoblastic shell H) Nitabuch s layer I) Decidua

ance are found in Nitabuch s layer (13) and in the intercellular deposits of the cell islands cell columns and shell (13 14) This does not necessarily mean that the composition of the granular deposit in all those sites is identical In all locations however the granular masses are mixed with varying amounts of fibrillar material with the appear ance of fibrin

In the present study immunohistochemical methods were applied to placentas at different stages of pregnancy in order to obtain further information about the nature of the various deposits The presence or absence of fibrin and plasma proteins was demonstrated by applying antisera against human fibrin fibrinogen albumin and γ globulin

MATERIALS AND METHODS

Tissue specimens Five mature and 5 immature placentas from normal pregnancies were examined The immature placentas were obtained from physically healthy women whose pregnancies were interrupted on psychiatric indications after 8 to 24 weeks gestation (menstrual age) Immediately after removal the placentas were gently rinsed in 0 9 per cent NaCl solution and cut in slices Pieces for study were taken from the central and peripheral parts of the placentas Some pieces were frozen in a dry ice/ethyl alcohol mixture at —70 C and then stored at —20 C Other specimens were placed directly in the freezer at —20 C, and some pieces were frozen by CO_2 gas on a freezing stage for immediate sectioning All prepara tive procedures gave similar results

For comparison a fresh endocardial thrombus from a patient who died from myocardial infarction was frozen by CO gas and treated in the same way as the placentas

Sectioning The specimens were sectioned at 6–8 microns in a Tissue Tek Cryo stat (Ames Lab Tek) Alternate sections were prepared for immunofluorescence study and for light microscopy

Conventional histological techniques Sections were stained with

(1) Haematoxylin azo phloxine (HP)
(2) Lendrum s Martius scarlet blue method (MSB)
(3) Mallory s phosphotungstic acid haematoxylin method (PTAH)

For the MSB staining sections were fixed in ether/ethyl alcohol (1 1) for approximately one hour hydrated through alcohols to water postfixed in 8 per cent formaldehyde with 5 per cent mercuric chloride for 3 days and stained according to Lendrum et al (9) For the PTAH staining sections were fixed in ether/ethyl alcohol for approximately one hour hydrated through alcohols to water postfixed in Zenker s fluid over night and stained with the technique of Mallory (11)

Immunofluorescence Technique

a) *Antisera* The following commercial antisera (Hyland Laboratories Los Angeles California U S A) were used

rabbit anti human fibrin
rabbit anti human fibrin labelled with fluorescein isothiocyanate (FITC)
rabbit anti human fibrinogen
rabbit anti human fibrinogen labelled with FITC
rabbit anti human albumin
rabbit anti human albumin labelled with FITC
rabbit anti human γG globulin labelled with FITC
goat anti rabbit γ globulin labelled with FITC

By immunoelectrophoresis against normal human plasma antisera against fibrin showed one precipitation line in the β region Anti fibrinogen serum showed a distinct line in the same position and a very faint line probably corresponding to α macroglobulin Anti albumin showed only one precipitation line Anti γG globulin showed a distinct line corresponding to γG globulin and a faint one to γA globulin

By gel diffusion tests anti fibrinogen serum showed one precipitation line against plasma diluted 1 2 and 1 10 and no line against normal human serum diluted 1 2 and 1 10 Anti albumin serum showed one precipitation line against human serum albumin (1 mg/ml) and normal human serum diluted 1 5 and 1 20

Anti γG globulin serum showed precipitation lines against γG globulin (1 mg/ml) γM globulin (1 mg/ml) and pepsin split γG (1 mg/ml) this indicated that the antiserum contained antibodies to γG globulin as well as to other γ globulins The anti γG serum will therefore be designated as anti γ globulin

The amount of specific antibody and the degree of conjugation was not determined for the different antisera Therefore when the reactions of the different antisera are compared the degree of fluorescence is not proportional to the concentration of the corresponding antigen Significant differences in protein concentration was indicated however when a single section showed varying intensity of fluorescence in different areas

b) *Staining technique* After sectioning staining was performed on the same day using either the direct method (9) or in some sections the "sandwich method (21) The sera were applied undiluted and diluted 1 10 with 0 9 per cent NaCl

c) *Microscopy* Fluorescence microscopy was performed with a Leitz Orthomat microscope using UG 1 and BG 38 filtres Photographs were taken with Agfa Isopan IFF film (15 din)

Control Experiments

1 *Autofluorescence* Unstained sections showed scarcely visible autofluorescence of a grey greenish colour different from the light apple green fluorescence of FITC

2 *Non specific staining* There was n binding of goat anti rabbit γ globulin labelled with FITC

3 *Adsorption* Anti fibrin and anti fibrinogen labelled with FITC were adsorbed with equal amounts 0 6 per cent purified fibrinogen (Kabi Stockholm Sweden)

Anti albumin labelled with FITC was adsorbed with equal amounts of 1 per cent human serum albumin (Kabi Stockholm Sweden) By gel diffusion tests anti fibrinogen labelled with FITC adsorbed with fibrinogen showed a precipitation line against anti fibrinogen labelled with FITC and no line against normal human plasma diluted 1 ? and 1 10 Anti albumin labelled with FITC adsorbed with albumin showed one precipitation line against anti albumin labelled with FITC. The anti fibrinogen and anti albumin sera were therefore adsorbed in excess Sections treated with adsorbed sera showed no fluorescence except for the auto fluorescence

4 *Staining of fibrin and fibrinogen* Fibrin clots were prepared from purified fibrinogen Fibrinogen precipitates were made from citrated plasma Both preparations were stained with anti fibrin and anti fibrinogen labelled with FITC. A detailed description of these experiments have been given in another paper (1a) Cross reactions for fibrin and fibrinogen were obtained for both Fibrin and fibrinogen can therefore not be differentiated with these methods and the term fibrin/fibrinogen will be used in the following text Fibrinogen precipitated from citrated plasma showed binding of anti albumin labelled with FITC, and mostly at the surface Fibrin clots formed from purified fibrinogen showed no binding of anti albumin labelled with FITC

RESULTS

The direct method and the sandwich method gave the same results in the localization of the fluorescence although more strong with the sandwich method

The fluorescence with both undiluted and diluted (1 10) antisera appeared in the same areas

In the following binding of anti fibrin and anti fibrinogen labelled with FITC will be designated as "binding of anti fibrin/fibrinogen Red stain with the MSB method and bluish black stain with the PTAH method are typical reactions of fibrin and are designated as positive

Binding of anti fibrin/fibrinogen was seen throughout the deposits with a distribution similar to that of the red colour seen in haematoxylin azo phloxine preparations Generally the deposits gave a positive reaction with the MSB and PTAH methods but within more restricted areas PTAH tended to give a positive stain to the fibrillar component of the deposits only whereas MSB gave a positive reaction to some of the homogeneous masses as well Compared with the results obtained in paraffin sections both the MSB and PTAH methods applied to frozen sections tended to give positive stain in less extensive areas (13 14 16)

Binding of Anti Fibrin/Fibrinogen

In the endocardial thrombus binding of anti fibrin/fibrinogen was seen throughout all layers but was particularly prominent at the surface (Fig 2) and at the base towards the endocardium In the MSB and PTAH preparations no obvious differences in the extent of positive stain was seen and both were similar to the distribution of anti body binding

For comparison a fresh endocardial thrombus from a patient who died from myocardial infarction was frozen by CO gas and treated in the same way as the placentas

Sectioning The specimens were sectioned at 6-8 microns in a Tissue Tek Cryo stat (Ames Lab Tek) Alternate sections were prepared for immunofluorescence study and for light microscopy

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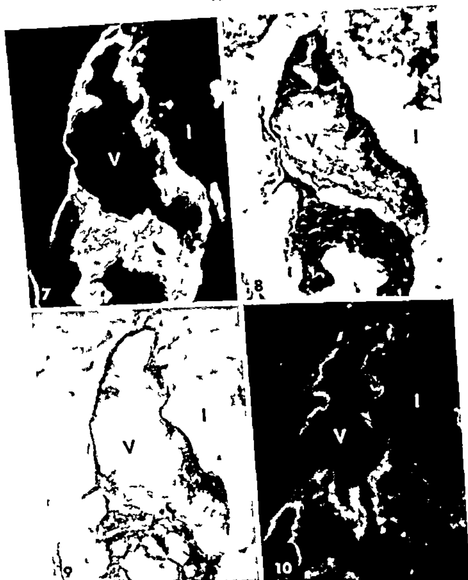
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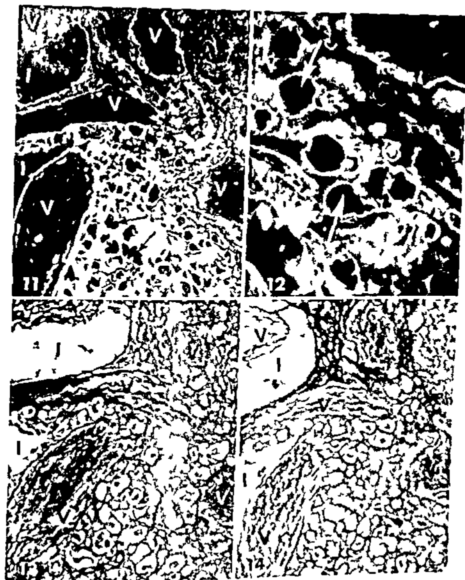
Figs 7–10

Fig 7 Placenta at 1? weeks Anti human fibrin labelled with FITC. An extensive deposit surrounding a stem villus (V) exhibits specific fluorescence Inter illous space I × 100

Fig 8 Placenta at 1 weeks Parallel section of that shown in Fig 7 Positive stain appears black Villus V Inte villous space I MSB × 100

Fig 9 Pl centa at 12 weeks Parallel section of that shown in Fig 8 Positive tain appears black PTAH × 100

Fig 10 Placenta at 1? weeks Anti human albumin labelled with FITC Parallel ection of that shown in Fig 9 Specific fluorescence at the periphery of the deposit against the intervillous space (I) and against the villus (V) × 100



Figs 11–14

Fig 11 Placenta at term Anti human fibrin labelled with FITC The sub horionic continuous mass (Langhan layer) exhibit specific flu rescence which is most pronounced at the periphery of the masses and a a halo around pro liferating cytotrophobla ti cells which present themselves as small dark defects (arrows) Larger defects are areas f fibrous villi (V) and intervillous space (I) × 100

Fig 12 Placenta at term Anti human fibrin labelled with FITC Higher magnification of Fig 11 Rounded lark d fects in areas of proliferating cyto trophoblastic cells (arrows) × 400

Fig 13 Placenta at term Parallel ection of that shown in Fig 11 I'ositive stain appears darl grey Collagen fibres black Fibrous villi V Intervillous space I MSB × 100

Fig 14 Placenta at term Parallel ection of that shown in Fig 13 I itive stain of fibrillar material appearing black Fibrous villi V Intervill us space I PTAH × 100

and PTAH although the stained material was not uniformly distributed (Figs 5 and 6)

Particularly in the mature placenta larger deposits were observed on the villi and the chorion plate (Figs 7 and 11) A narrow zone along the periphery gave a brighter fluorescence of anti fibrin/fibrinogen than the central parts (Fig 11) Where proliferating cytotrophoblastic cells were found within the deposits, a halo of more pronounced fluorescence was often seen around the cells (Fig 12) MSB resulted in a positive stain in great areas of the larger deposits (Figs 8 and 13) whereas PTAH gave a positive reaction in patches and streaks (Figs 9 and 14)

Both in Rohr s stria and in Nitabuch s layer a bright anti fibrin/fibrinogen fluorescence was produced throughout (Figs 15 and 18) Further in MSB and PTAH preparations abundant positive stain was seen in both layers in certain instances the positive stain was nearly as extensive as the fluorescence in the antibody preparations (Figs 17 and 20)

In the cell islands cytotrophoblastic cell columns and shell the binding of anti fibrin/fibrinogen was found intercellulary at all gestational stages (Fig 21) However the fluorescence particularly of the shell seemed to be more intensive in the mature than in the immature placentas when compared to the fluorescence of the deposits on the villi in the same section In most areas of the islands columns and shell the fluorescence was more pronounced near the surface of the deposits towards the intervillous space than in the inner parts As in the larger deposits of the villi and in Langhans layer a halo of intense fluorescence was surrounding the cells (Fig 21) Fluorescent material occurred only rarely in the cytoplasm of these cells MSB and PTAH gave a positive stain in some regions of the intercellular material of the cell islands cytotrophoblastic cell columns and shell but was negative in other areas In the cell columns and cell islands the pattern of MSB staining was in accordance with the binding of anti fibrin/fibrinogen on positive stain near the intervillous space and only scattered areas of positive stain in the central regions (Fig 22) The PTAH method showed a similar distribution of the positive stain but was in most cases completely negative in central areas (Fig 23) In the cytotrophoblastic shell both MSB and PTAH were usually negative

In the decidua only a very faint anti fibrin/fibrinogen fluorescence was present between the cells MSB and PTAH were negative

Binding of Anti Albumin

In the endocardial thrombus anti albumin (Fig 3) fluorescence was only found on the surface and was not present in the inner parts

A similar distribution was seen in the deposits on the syncytium of the villi and the chorion plate Anti albumin fluorescence was seen in

a narrow zone along the periphery of the masses both towards the maternal blood space and at the inner border against the villus or chorion plate but was occasionally present within the deposits as in the case shown in Fig 10

In Rohr's stria anti albumin gave only a faint fluorescence (Fig 16) as compared with that along the surface of the deposits on the villi In Nitabuch's layer only a weak fluorescence was occasionally seen (Fig 19)

In the cell islands cell columns and shell anti albumin gave a very



faint fluorescence in the intercellular matrix occasionally patchy areas with a brighter fluorescence were seen (Fig 24)

Intercellularly in the decidua only a faint anti albumin fluorescence was seen

Binding of Anti γ Globulin

The binding of anti γ globulin labelled with FITC nearly always showed the same localization as anti albumin labelled with FITC

In Nitabuch s layer anti γ globulin fluorescence was not present

DISCUSSION

The immunohistochemical techniques are sensitive and selective methods for the demonstration of plasma proteins in tissue sections

Many standard methods have been worked out for the staining of fibrin It is a common observation however that the immunohisto chemical methods give a more extensive distribution of positive fibrin stain than the standard tinctorial methods (3 6 8 22) This may partly be due to cross reactions between fibrin and anti fibrinogen and be tween fibrinogen and anti fibrin (15 20) Furthermore it is known that anti fibrin and anti fibrinogen react not only with fibrin and fibrinogen but also with some of the split products and degradation products of these proteins (12 18) Therefore the immunohisto

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Figs 15–20

Fig 15 Placenta at 24 weeks Anti human fibrin labelled with FITC. Rohr s stria (R) exhibits specific fluore cence *Intervillous* space I Cytotrophoblastic shell C × 400

Fig 16 Placenta at 24 weeks Anti human albumin labelled with FITC. Parallel section of that shown in *Fig* 15 *Faint fluorescence* as a halo around the cyt trophoblastic cells (arrows) Rohr s stria R Intervillous space I Cyt trophoblastic shell C × 400

Fig 17 Placenta at 24 weeks Parallel section of that shown in Fig 16 Positive stain of the deposit at the surface (arrows) At upper part a villus (V) Intervillous space I Cytotrophoblastic shell C. MSB × 400

Fig 18 Placenta at 24 weeks *Anti human* fibrin labelled with FITC Nit buch s layer exhibits specific fluorescence Cytotrophoblastic shell C. Decidua D × 400

Fig 19 Placenta at 24 weeks *Anti human* albumin labelled with FITC. Parallel section of that sh n in Fig 18 Area of Nitabuch s layer N Cytotropho blastic hell C. Decidua D × 400

Fig 20 Placenta at 24 weeks Parallel section of that shown in Fig 19 Positive stain of Nitabuch s la er appears black. Cytotrophoblastic shell C. Decidua D PTAH × 400



Figs 21–2

Fig 21 Placenta at term Anti human fibrin labelled with FITC Fluorescence in the intercellular material of a cell island Rounded dark defects in areas of the cells (arrows) Intervillous space I × 100

Fig 22 Placenta at term Parallel section of that shown in Fig 21 Positive stain of the intercellular material peripherally and small patches centrally (arrows) Intervillous space I MSB × 100

Fig 23 Placenta at term Parallel section of that shown in Fig 22 Positive stain appear black at the periphery of the cell island PTAH × 100

Fig 24 Placenta at term Anti human albumin labelled with FITC Parallel section of that shown in Fig 23 Small patches with bright fluorescence intercellulary Intervillous space I × 100

chemical techniques demonstrate fibrin fibrinogen and some of their derivatives whereas conventional histological techniques stain fibrin (5 15) and fibrinogen (15) only under certain conditions as shown in *in vitro* experiments

Furthermore the immunohistochemical method is more specific

because standard tinctorial methods may give a positive stain of substances not related to fibrin *e g* a positive stain of γM globulin by MSB (15)

The small deposits on the syncytium gave a bright fluorescence with the anti fibrin/fibrinogen preparations

In light and electron microscopical studies of human placentas these early small deposits in relation to the villi on the chorion plate (16) and in Rohr s stria (13) were found to have the structure of platelet thrombi at various stages of transformation to fibrin thrombi The present evidence of the localization of fibrin/fibrinogen in these areas is in agreement with the previous results

Judged by the findings in the present study the large intervillous deposits particularly found in the mature placentas contained a considerable amount of fibrin fibrinogen and/or their derivatives This fits with the theory that the deposits are composed of aged fibrin assumed to be remnants of old platelet fibrin thrombi (16)

When stained with anti fibrin/fibrinogen the brighter fluorescence along the edges of these masses need not reflect any differences in the concentration of fibrin as *in vitro* immunohistochemical studies of fibrin clots revealed a similar staining pattern of the fibrin strands (5) However the concentration at the surface might be due to non specific reactions but the negative stain with the antiserum adsorbed in excess excludes this theory

Occasionally fibrin/fibrinogen was observed within the cytoplasm of the trophoblastic cells underlying the intervillous masses This may well be a sign of cell injury (7) and it is in accordance with the electron microscopical finding of intracellular fibrin strands at these sites (16)

Unlike the deposits bordering the intervillous space Nitabuch s layer and the intercellular materials of the cell islands cell columns and shell are not of thrombogenic origin as platelets were not found in these locations (13 14 23)

In Nitabuch s layer a constant and strong fluorescence for fibrin/fibrinogen suggests that substantial amounts of fibrin fibrinogen and/or their derivatives are present This is in good agreement with the positive MSB and PTAH stain and previous findings using other conventional techniques (10 17 19) By electron microscopy fibrils indicative of fibrin are found intermingled with granular masses and cell debris (13)

Deposition of fibrin/fibrinogen was found throughout the intercellular deposits of the cell islands cell columns and shell Although scattered fibrin fibrils were seen ultrastructurally in the intercellular deposits of these cell collections the major components were granular masses and cell debris (13 14) The binding of anti fibrin/fibrinogen to areas mostly corresponding to granular masses ultrastructurally is therefore not to any great extent due to the presence of newly formed

fibrin but is probably related either to fibrinogen or aged fibrin (16), *i e* a material similar to that of the larger thrombogenic deposits bordering the intervillous space

Anti albumin gave fluorescence only at the periphery of the intervillous deposits and Langhans layer This is contrary to *Brosko et al* (1) who found albumin throughout the intervillous deposits In the present study however, albumin was also located only at the periphery of the endocardial thrombus The deposition of albumin therefore seems to be a non specific adsorption to the surface of thrombi That the fluorescence is contingent upon a specific immunological reaction was shown by a negative stain with the antiserum adsorbed in excess

In the intercellular deposits of the cell islands cell columns and shell anti albumin gave a very faint fluorescence and occasionally patchy areas with a brighter fluorescence This was in contrast to the bright and even fluorescence of fibrin/fibrinogen throughout the deposits This could mean that fibrinogen or its derivatives constitute a component of the deposits more important than albumin At no stage of pregnancy does the placenta synthesize albumin or γ globulin (4) but the possibility exists that these cells may synthesize fibrinogen as they exhibit secretory activity ultrastructurally (13 14) However it is more likely that the fibrinogen and albumin in these locations originate from the maternal plasma leaking from the intervillous space The fact that fibrinogen or its derivatives were more extensively distributed than albumin may be explained by the particularly easy precipitation of fibrinogen or by formation of fibrin

Anti γ globulin fluorescence nearly always showed the same localization as anti albumin fluorescence The controls as regards the anti γ globulin serum are considered to be insufficient Staining with anti γ globulin labelled with FITC may therefore be contingent upon non specific reactions However areas not stained with anti γ globulin labelled with FITC *e g* Nitabuch s layer most likely do not contain substantial amounts of γ globulin The lack of immunoglobulins in Nitabuch s layer probably indicates that this zone does not contain immunological reaction products

SUMMARY

The deposits in the normal human placenta at various stages of gestation were studied using FITC labelled rabbit antisera against human fibrin fibrinogen albumin and γ globulin

Fibrin or fibrinogen was found to be present throughout the deposits on the syncytium of the villi and the chorion plate in Rohr s stria and in Nitabuch s layer Fibrin or fibrinogen was also present in the intercellular material of the cell islands and cytotrophoblastic cell columns at all stages of pregnancy and in the intercellular material of the cytotrophoblastic shell particularly at an advanced gestational age

Albumin was found to be present along the periphery of the deposits on the syncytium In the intercellular deposits of the cell islands cell columns and shell FITC labelled anti albumin gave a very faint fluorescence and occasionally patchy areas with a brighter fluorescence Only traces of albumin was present in Rohr s stria and Nitabuch s layer

γ globulins were not present in Nitabuch s layer

The findings are in agreement with previous evidence indicating that deposits on the syncytium and Rohr s stria develop from platelet fibrin thrombi The intercellular deposits of the cell islands cell columns, shell and Nitabuch s layer are possibly fibrin or fibrinogen precipitated from maternal plasma seeping through the tissue from the intervillous space

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SEX DIFFERENCE IN THE GROWTH OF A STRAIN SPECIFIC MOUSE TUMOUR, TA3

By

F HARTVEIT

Received 1 xi 68

Sex differences in the growth of transplantable tumours are said to be rare (see *Snell* 1953) but have recently been encountered at this Institute Thus the Ehrlich ascites carcinoma a non specific mouse tumour grows better subcutaneously in male mice (*Hartveit* 1962 b) in which the survival time is also less than in females (*Hartveit* 1962 a) and the strain specific TA3 carcinoma grows better in strain A males than females (*Thunold* 1966)

The following experiment again demonstrates this sex-difference in the growth of TA3 in A mice and also in their F_1 hybrids and further relates this finding to the survival time of the animals

One subcutaneous injection of 0 25 ml of a saline suspension of TA3 cells made up from a solid subcutaneous transplant in a male A mouse was given to each of 6 male and 6 female A mice and to a similar number of F_1 hybrids (strain A male crossed with females of our closed colony) The sum of the two greatest tumour diameters was measured during growth and the survival time of the mice recorded

TABLE 1
Tumour Size and Survival Time in A and F_1 Hybrid Mice with TA3 Carcinoma

Sex	Tumour size (mm) ± SD		Survival time (days) ± SD	
	A mice	F_1 mice	A mice	F mice
Male	62 5 ± 9 4	57 0 ± 1[illegible] 6	48 [illegible] ± 3 4	50 0 ± 4 3
Female	37 0 ± 11 7	[illegible]5 8 ± 14 9	59 4 ± 3 9	75 8 ± 1 8

See text.

The tumour measurements at 24 days (i e before the first mouse died) are shown in the Table 1 The tumour grew better in male than female

Research Fellow Norwegian Cancer Society

I am indebted to Professor *G Klein* for supplying our original transplant of the TA3 carcinoma

mice of both types (0.01>P>0.001). The difference between A and F_1 mice of the same sex was not significant. The survival time is also shown in the table. The male mice died earlier than the females of both types (A: 0.01>P>0.001; F_1: 0.001>P). The difference in the survival time of the A and F_1 male mice was not significant, but the female F_1 mice outlived the A females (0.001>P).

The present findings thus confirm that *TA3 grows better in male than in female A mice*, and extends this observation to *their F_1 hybrids*. The possibility that this sex difference in tumour growth is due to a direct *Eichwald Slimser* effect (1955) is ruled out as TA3 arose in a female mouse and the more remote possibility of its being due to an Eichwald Slimser like effect due to the presence of male host cells injected with the tumour cells is excluded by Thunold's experiment in which the tumour was transplanted from a female mouse. The sex difference is therefore likely to be due to a difference in host response to the tumour cells themselves.

The findings that *tumour growth is similar in A and F_1 mice*, and that *the survival time of the F_1 mice was either similar to or greater than that of the A mice* are at variance with the general impression that a tumour may grow better in F_1 hybrids of its strain of origin (*Little* 1956). It is argued that the increased vitality of such hybrids provides a more favourable environment for tumour growth. In the present case the argument that hybrid vigour may be accompanied by a more intense immune response leading to inhibition of tumour growth would seem more pertinent as the effect was seen in female mice which may show a more intense immune response than males (*Halpern* 1964; *Hartveit* 1965). This latter explanation presupposes that TA3 carries with it some antigenic factor, e.g. tumour specific antigen or virus, that enables the mice to react against the otherwise isologous tumour cells.

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MORPHOLOGIC EFFECTS OF ETHIONINE ON THE PANCREAS OF THE CHINESE HAMSTER

A Light and Electron Microscopic Study of Degenerative Changes

By

LENNART BOQUIST

Received 25.x.68

Ethionine is known to produce destruction of the acinar pancreatic parenchyma (*cf. Farber* 1959, *Seifert & Gieseking* 1961, *Ekholm et al.* 1962, *Herman & Fitzgerald* 1962, *House et al.* 1963) and to induce fatty liver (*cf. Farber* 1967) and hepatic tumours (*Popper et al.* 1953). This compound may also evoke morphologic alterations in salivary (*Loring & Hartley* 1955, *Ulmansky & Ungar* 1967) and lacrimal glands (*Benson* 1964), gastrointestinal tract (*Loring & Hartley* 1957, *Kaufman et al.* 1962), testis (*Goldberg et al.* 1959) and kidneys (*Alvi ouri & Warren* 1954). The damages induced by ethionine in the pancreas bear resemblance to those seen during protein deprivation (*Weisblum et al.* 1962). In animals treated with ethionine the pancreatic islets are usually said to be preserved and even to show some hyperplasia in later stages (*Farber* 1959).

The present study was initiated as an extension of our previous work on various kinds of degenerative and regenerative islet changes in the Chinese hamster (*Boquist* 1968a, b, c and 1969a). Its main purpose was to examine whether agents known to damage the acinar pancreatic parenchyma also could affect the islet cells. To the best of our knowledge there are no previous reports on the morphologic effects of ethionine in the Chinese hamster. The present investigation is confined to the degenerative pancreatic alterations occurring in this species during the first 2 weeks after the administration of ethionine. A report on the ensuing regenerative changes will be published separately (*Boquist* 1969b).

MATERIAL AND METHODS

Fourty two non diabetic adult Chinese hamsters of both sexes from 3 months to about years of age were used. They were kept on a standard laboratory diet and water *ad libitum* and were housed in individual cages in animal rooms at a temper-

This work was supported by grants from the Swedish Medical Research Council (Project No. B69-12X-718-04A).

ature of 20–22 °C. Twelve of these animals served as controls and received daily intraperitoneal injections of 1 ml of saline. The other 30 hamsters got daily injections of DL ethionine (Sigma Chemical Company, St. Louis, U.S.A.) that was prepared daily by dissolving the amount to be used in saline heated to 50 °C. The ethionine was administered intraperitoneally at a dose of 0.5 g/kg body weight. Two control and five ethionine treated hamsters were sacrificed at 1, 2, 4, 7, 10 and 14 days after the beginning of the experiments. There were no animals dying spontaneously. At sacrifice, specimens were taken from the pancreas for light and electron microscopic examination.

For light microscopy, pancreatic slices were fixed in 10 per cent formalin or Bouin's fixative. The following stains were used: van Gieson's stain, haematoxylin-eosin, aldehyde fuchsin in the modification by Maske (1955), chrome alum haematoxylin counterstained with ponceau fuchsin, silver impregnation according to Hellerström & Hellman (1960) and periodic acid Schiff stain.

For electron microscopy, multiple pancreatic blocks of about 1 mm³ were fixed in 1 per cent osmium tetroxide in 0.34 M veronal acetate buffer adjusted to pH 7.4. The fixation was carried out at 0–4 °C for 2 hours. After fixation the slices were rinsed in physiological saline, dehydrated in rising concentrations of ethanol and embedded in Epon 812 (Luft 1961). In order to find appropriate areas for the thin sections, thick (1 μ) sections were stained with toluidine blue and studied in a light microscope. The sections were cut on an LKB Ultrotome III and were stained with uranyl acetate and lead citrate. Examination of the sections were performed in a Siemens Elmiskop I A and a Zeiss EM 9 (at 60–80 kV) and electron micrographs were taken at original magnifications of 2 000–80 000.

RESULTS

Light Microscopy

Control Animals

The morphology of the exocrine pancreas appeared to conform to that of other mammals and there were no degenerative or regenerative alterations (Fig. 1). The islets were normal.

Ethionine Treated Animals

From the second day there were changes of the pancreas in all the animals. These changes varied, however, in different parts of the same pancreas and areas with marked lesions and those with normal appearance occurred close to each other. There were also variations between animals treated alike. No sex or age differences were found.

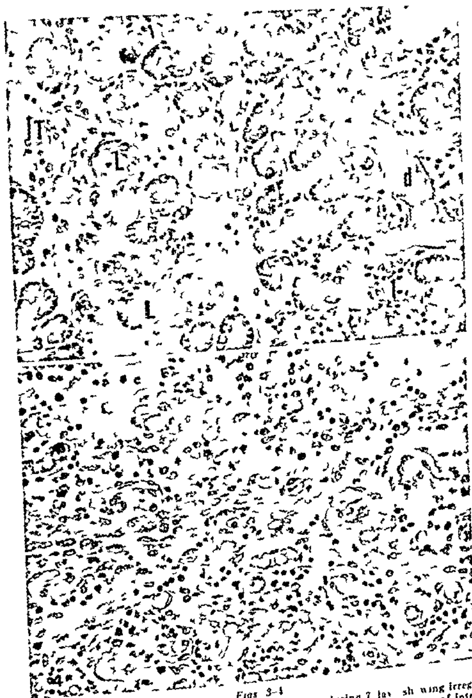
After 24 hours' ethionine exposure scattered inflammatory cells were found in the pancreas of some animals. At 2 days the acinar cells showed decrease of basal basophilia. There was diffuse infiltration of polymorphonuclear leucocytes, plasma cells and lymphocytes. From the 4th day there were nuclear pyknosis and cytoplasmic vacuolization (Fig. 2)

Figs. 1–2.

Fig. 1. Pancreas of control hamster showing acini with small lumina. The connective tissue component is inconspicuous. Aldehyde fuchsin stain. × 400.

Fig. 2. Pancreas from Chinese hamster treated with ethionine during 4 days demonstrating decreased staining affinity of the acinar cells (A). There are cytoplasmic vacuoles (v), ductules (d) and some lymphocytes. Aldehyde fuchsin stain. × 400.


d
A



Figs 3–4

Fig 3 Pancreas from hamster treated with ethionine during 7 days showing irregular and dilated acinar lumina (L), inflammatory cells (I), increase of interstitial tissue (IT) and longitudinal section of ductule (d). van Gieson's stain × 250

Fig 4 Atrophied acinar tissue of Chinese hamster after 10 days of ethionine exposure showing polymorphonuclear leucocytes, lymphocytes, vacuolised cells and interstitial oedema. van Gieson's stain × 250



Fig 5

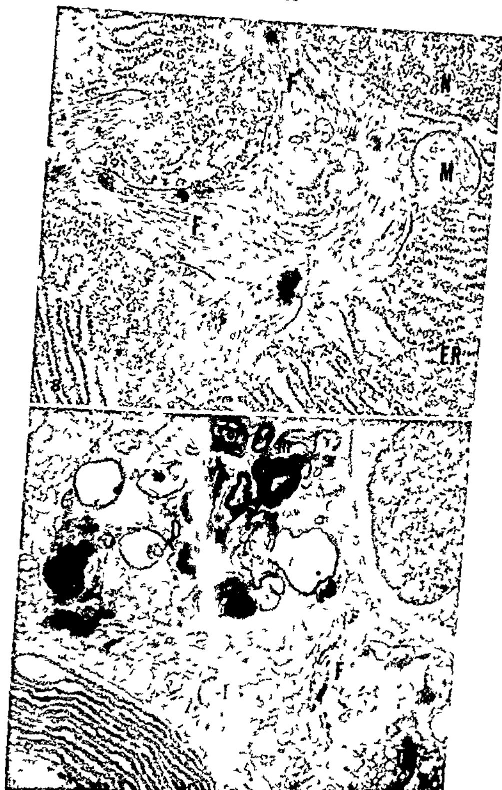
Pancreatic islet from Chinese hamster treated with ethionine during 10 days containing central β cells (β) There are large peripheral α -cells (α) exhibiting large nuclei and distinct nucleoli and well demarcated cytoplasm Swollen and necrotic cells (n) probably α -cells also occur In the exocrine portion there is swelling and decreased staining affinity of some acinar cells (A) Aldehyde fuchsin stain × 300

Many cells showed complete loss of basal basophilia and there were cells with eosinophilic bodies in the cytoplasm In the acini there were cells of varying size and staining affinity as well as disintegrated cells In the acinar lumina there were dark masses and eosinophilic bodies At 7 days there were irregular acini with dilated and irregular lumina (Fig 3) that sometimes were filled with dark masses and light amorphous material Atrophy of the acinar parenchyma and replacement by adipose and fibrous tissue occurred mainly at 10 and 14 days In the atrophied parenchyma unaffected acini could be seen Infiltration of inflammatory cells and interstitial oedema were found in areas of atrophy (Fig 4)

In the *ducts and ductules* there were at all observation times dark masses and occasionally light amorphous material was found in the ductules In areas of atrophy there were numerous ductules and a moderate amount of ducts Some of these appeared to be dilated In the ducts there were sometimes single or multiple goblet cells The ductule cells were unaffected The only alteration observed in the duct cells was the occasional occurrence of crystalline material

The *vessels* in the exocrine pancreas sometimes showed rather thick basement membranes The *nerves* were unaffected

In the *islets* the β cells often showed degranulation At 10 and 14 days there were a few islets with changes of the α_2 cells (Fig 5) Thus


F
N
M
F
ER
F

There were numerous cytoplasmic bodies of varying size and appearance In cells with or without other changes there were bodies consisting of fibrillar elements with varying arrangement (Fig 8) These were tentatively called fibrillar bodies Enclosed within them there were sometimes small electron dense rounded or irregular particles The fibrillar bodies occasionally seemed to be connected with mitochondria Membrane-enclosed fibrillar bodies were also encountered

In addition to these cytoplasmic bodies there were more electron dense and irregular structures of varying size Myelin figures and focal accumulations of degenerated cellular components often occurred (Fig 9) There was also necrosis of whole cells

The architecture of the acini was altered There were areas with acinar atrophy and replacement by adipose and fibrous tissue In the acinar lumina there were fibrillar material moderately electron dense amorphous masses and structures suggestive of being degenerative cellular components Thus these structures showed varying electron density and contained particles reminiscent of membranes and granules

Duct system The lumina of the duct system contained moderately electron dense amorphous masses There were apical blebs in the ductule cells Crystalline material was occasionally found in the duct cells (Fig 10) In the ducts there were sometimes goblet cells (Fig 11) In a few of these there was also crystalline material

Pancreatic islets Degranulation of the β cells was often found from the second day At 10 and 14 days there were a few islets with changes of the α cells Thus some of these cells showed prominent nucleoli well developed endoplasmic reticulum and Golgi complex as well as numerous mitochondria and secretory granules Other α cells disclosed swelling and disintegration of the mitochondria and cytoplasmic vacuolization There were also cells with marked degenerative changes with disintegration of the cytoplasm and the cellular organelles (Fig 12) The α_1 cells and the agranular cells were unaffected Sometimes the islet capillaries showed rather thick basement membranes

DISCUSSION

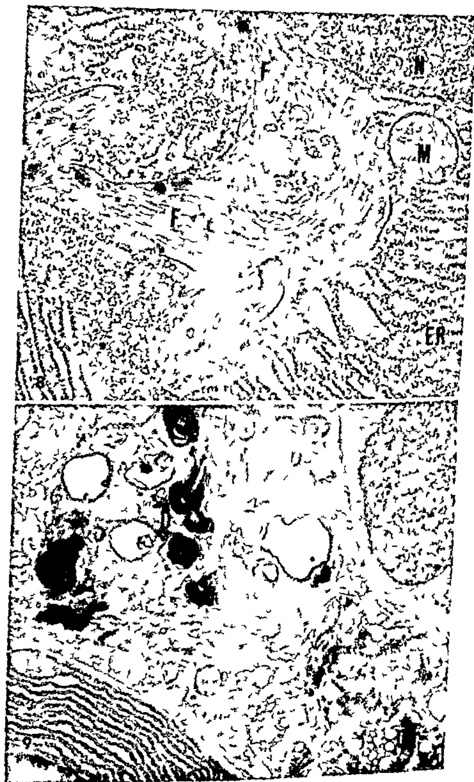
Many of the studies with ethionine have utilized animals fed stock or special diets for periods of days to weeks One of the most obvious

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Figs 8–9

Fig 8 Portion of acinar cell of hamster after 4 days of ethionine treatment showing fibrillar bodies (F) containing small electron dense particles endoplasmic reticulum (ER) a mitochondrion (M) and nucleus (N) × 34 000

Fig 9 Pancreas from Chinese hamster treated with ethionine during 7 days showing an acinar cell with accumulations of irregular dense vesicular or membraneous structures (D) some of these are myelin like (m) There are also fibrillar bodies (F) × 16 000


F
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DISCUSSION

Many of the studies with ethionine have utilized animals fed stock or special diets for periods of days to weeks One of the most obvious

Fig 8 [illegible] fibrillar [illegible] days [illegible] ethionine treatment sh wing reticulum [illegible] particles endoplasmic

Fig 9 Pancreas [illegible] leus (N) × 31 000 an acinar cell [illegible] ethionine during 7 d ys sh wing [illegible] dense vesicular [illegible] mem fibrillar bodi [illegible] myelin like (m) There are als[illegible]


d
GC
GC
10
GC



Fig 1?

Peripheral portion of pancreatic islet fr m Chine e hamster treated with ethionine during 10 days showing cells with degenerative changes (n) In some of these cells there are secretory granules of α_2 type (α) There is one cell with sparse granulation probably a degranulated β cells (β) in which there are no obvious degenerative changes A capillary (C) with thick basement membrane (BM) is also seen × 3 000

uncertainties in many such studies is the large and variable decrease in food intake of animals given ethionine as compared to control animals (cf *Farber* 1967) If ethionine is given parenterally it seems to be more easy to get proper dosage than if it is given in the diet In rats no clear cut difference is found whether or not ethionine is administered

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Figs 10–11

Fig 10 Pancreatic duct of Chinese hamster treated with ethionine during 7 days demonstrating duct cells which contain electron dense particles apparently f crystalline nature (arrows) In the duct there are also goblet cells (GC) In one of the e the contents are discharged (d) into the lumen through the ruptured cell membrane × 3000

Fig 11 Portion of pan reatic duct of hamster after 7 days ethionine exposure showing a goblet cell (GC) In this cell as well a in the neighbouring duct cells there is electron dense apparently crystalline material (arrows) × 17 000


GC
d
GC
10
GC
11

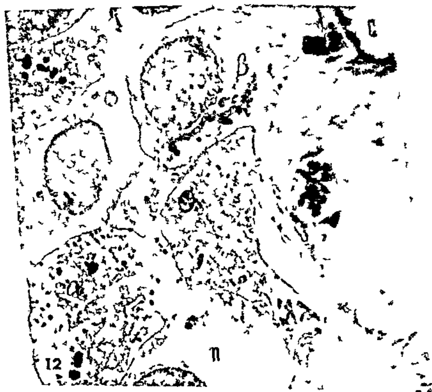


Fig 12

P ripheral porti n of pancreati islet from Chinese hamster treated w during 10 days sho ving cells with d generative changes (n) In s me of the there are secretory granules of α type (α) There is one cell with sparse g prob bly a degranulated β cells (β) in which there are n obvious degen changes A capillary (C) with thick basement membrane (PM) is also seen × 3 0

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Fig 10 Pancreatic duct f Chinese hamster treated with ethionine during 7 days demonstrating duct cells which contain electron dense particles apparently of crystalline nature (arr ws) In the duct there are als goblet cells (CC) In one of these the contents are discharged (d) into the lumen through the ruptured cell membrane × 3000

F g 11 Porti n f pancreatic duct of hamster after 7 days ethionine exposure h wing a goblet cell (CC) In this cell as well as in the neighb uring duct cells there is electron dense apparently cr stalline material (arrows) × 17 000

directly into the blood stream (*Loring & Hartley* 1955). Intraperitoneal injections have been used in the rat for investigations of the effects on the pancreas by ethionine (*Loring & Hartley* 1955, *Herman & Fitzgerald* 1962). If animals receive ethionine during several days or longer the results are difficult to interpret because of the ability of the organism to adapt readily to this compound within a period of 2 or 3 weeks (cf. *Farber* 1967). On the other hand *House et al.* (1963) state that one to three weeks are required for complete action of ethionine in the golden hamster. The time factor is important in the interpretation of the significance of experimental observations with ethionine (*Farber* 1967). In studies of the effect of ethionine on ribonucleic acid synthesis in the liver it has been found that there is a fall in the incorporation of labelled precursors into RNA in acute experiments (*Farber et al.* 1964) but a stimulation of RNA synthesis in long time experiments (*Turner & Reid* 1964). There are differences in the effects if ethionine is administered prolonged or in a large single dose (cf. *Meldolesi* 1967). In the present work it was thought most appropriate to give the ethionine as daily intraperitoneal injections. By the use of various observation times it was hoped that difficulties in the interpretation because of the time factor would be diminished.

The experiments show that the administration of ethionine to the Chinese hamster evolves marked changes of the exocrine pancreas. Among these changes those exhibited by the endoplasmic reticulum were conspicuous and early occurring. It has been postulated that alterations of the endoplasmic reticulum belong to the earliest and most characteristic in ethionine treated animals (*Ekholm et al.* 1962, *Herman & Fitzgerald* 1962). Reduction of the number of ribosomes and altered organization of endoplasmic reticulum have been thought to be consistent with an involvement with RNA and protein metabolism (*Herman & Fitzgerald* 1962) and to be equivalent to the diminution of basophilic substance observed in the light microscope (*Ekholm et al.* 1962).

There are different opinions as to the effect of ethionine on the zymogen granules. Thus there are descriptions of increased (*Farber & Popper* 1950, *Edlund* 1962) or decreased (*Goldberg & Chaikoff* 1951) number of these organelles. The results of the present study seem to conform to those obtained by the latter authors. The structures interpreted as prozymogen granules showed membrane discontinuities conforming to those seen in pancreatic acinar cells of rabbits (*Shapiro & Lazarus* 1967).

Fibrillar bodies were often encountered. The nature of these is not known but it seems probable that they may be of degenerative nature and possibly originate from endoplasmic reticulum or mitochondria. In acinar cells of chicken cytoplasmic inclusions consisting of parallel tubular elements have been found (*di Stefano* 1967). Though these inclusions apparently not exactly conform to the fibrillar bodies

it is known that these cytoplasmic inclusions may occur in association with mitochondria and endoplasmic reticulum

Necrosis of whole acinar cells occurred in the ethionine treated hamsters There were also *focal accumulations of degenerated cellular* material including myelin structures This may denote degradation of part of a cell with possibilities for resorption or extrusion of the degenerative material (*cf Swift & Hruban* 1964) If so it seems that acinar cells with limited lesions may survive the effects of ethionine and possibly regenerate

The nature of the crystalline material sometimes seen in the duct cells is unknown and remains to be clarified The significance of the goblet cells in the ducts is not clear Though they have not been observed by us in the ducts of normal Chinese hamsters it cannot be excluded that they sometimes may occur normally in this species *Lev & Spicer* (1965) state that goblet cells in man only appear in the pancreatic ducts as they approach the duodenum As goblet cell metaplasia is found in damaged pancreas mainly in areas of scarring (*Walters* 1965) it seems however that the occurrence of goblet cells in the present study may be regarded as a secondary phenomenon to the ethionine induced lesions

Most works on the effects of ethionine on the pancreas have no reports of islet cell degeneration (*Farber* 1959) In the present study there were α cells showing prominent nucleoli endoplasmic reticulum Golgi complex as well as numerous mitochondria and secretory granules These alterations seem to indicate increased activity of these cells There were also a few α cells with marked degenerative changes In one study of ethionine treated rats the α cells showed swelling degranulation and hydropic degeneration whereas the β cells were unaffected This was thought to be due to reduced activity of tryptophan peroxidase as there is need for tryptophan in the synthesis of glucagon but not in that of insulin (*cf Seifert & Gieseking* 1961)

The significance of the seemingly haphazard distribution of the ethionine induced lesions with unaffected and atrophic acini close to each other is not known Such variations have been found also in ethionine treated rats (*Herman & Fitzgerald* 1962) It has been pointed out by *Fitzgerald* (1960) that it is very difficult if at all possible to destroy all acinar cells by ethionine and that these cells may survive even high doses of this compound

In the rat liver there is a sex difference in the response to ethionine and there is considerable evidence that the male rat metabolizes methionine and ethionine somewhat more slowly than does the female (*cf Farber* 1967) Sex differences as to the effect of ethionine on the pancreas in the golden hamster have been recorded (*House et al* 1963) In the present study sex differences were not found

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SUMMARY

DL-ethionine was administered intraperitoneally at a dose level of 0 5 g/kg body weight to 30 Chinese hamsters Twelve animals injected with saline were used as controls At pre determined intervals the animals were sacrificed and specimens were taken from the pancreas for light and electron microscopic examination of degenerative changes

In the light microscope the acinar cells showed decrease of basal basophilia cytoplasmic vacuoles and eosinophilic bodies The acinar lumina were dilated and irregular and contained light or dark masses and eosinophilic bodies There was atrophy of the acinar parenchyma with replacement by adipose and fibrous tissue Ultrastructurally the acinar cells demonstrated dilatation of the endoplasmic reticulum decreased number of ribosomes and zymogen granules disintegration of the mitochondria as well as the occurrence of cytoplasmic fibrillar bodies Focal accumulations of degenerated cellular components and necrosis of whole acinar cells were found In the ducts there were goblet cells and occasionally crystalline material occurred in the duct cells Degranulation of the β cells was found in the pancreatic islets In a few islets some α cells showed signs of increased activity whereas others disclosed marked degenerative changes The α_1 cells and the agranular cells were unaffected Thick basement membranes were sometimes seen in the vessels of the exocrine and endocrine pancreatic parenchyma

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SUMMARY

DL-ethionine was administered intraperitoneally at a dose level of 0.5 g/kg body weight to 30 Chinese hamsters. Twelve animals injected with saline were used as controls. At pre-determined intervals the animals were sacrificed and specimens were taken from the pancreas for light and electron microscopic examination of degenerative changes.

In the light microscope the acinar cells showed decrease of basal basophilia, cytoplasmic vacuoles and eosinophilic bodies. The acinar lumina were dilated and irregular and contained light or dark masses and eosinophilic bodies. There was atrophy of the acinar parenchyma with replacement by adipose and fibrous tissue. Ultrastructurally the acinar cells demonstrated dilatation of the endoplasmic reticulum, decreased number of ribosomes and zymogen granules, disintegration of the mitochondria as well as the occurrence of cytoplasmic fibrillar bodies. Local accumulations of degenerated cellular components and necrosis of whole acinar cells were found. In the ducts there were goblet cells and occasionally crystalline material occurred in the duct cells. Degranulation of the β cells was found in the pancreatic islets. In a few islets some α cells showed signs of increased activity, whereas others disclosed marked degenerative changes. The α cells and the agranular cells were unaffected. Thick basement membranes were sometimes seen in the vessels of the exocrine and endocrine pancreatic parenchyma.

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TABLE 1
Vaccination History of 324 Pre School Children

Children born in	Year of primary immunization against poliomyelitis 1961			1962			1963			1964			1965			1966			Vaccination not performed	Vaccination uncertain	Total
		3	4	2	3	4	2	3	4	2	3	4	2	3	4	2	3	4			
1960	1	13	23	[illegible]	22	3		[illegible]			2			2		1				3	77
1961		21			25	4		28	2		6					3			1	5	7[illegible]
196[illegible]					3		3	23		1	25			4					1	1	6[illegible]
1963											2[illegible]		4	18		1			1	5	5[illegible]
1964										1	2		[illegible]	34		8			1	1	53
Number of injections																					324

with imported inactivated vaccine During the following 8 year period the vaccinations were extended to all individuals Since 1958 solely inactivated vaccine manufactured in Sweden has been employed

The vaccination course recommended runs as follows Two injections of 1 ml of inactivated vaccine are given 1 to 2 months apart A third injection is administered 1 year after and a fourth 5 years after the first two doses

Plan of the Study

Altogether 27 day nurseries play schools and first grade school classes in Stockholm city were enroled These comprised close to 1100 children aged 2 to 7 years Questionnaires were distributed to the parents of the children Distribution and collection was carried out by aid of the personnel at the different units

The questions concerned the following How many poliovirus vaccine injections has the child received? In which years were they performed? Do you consider your data correct probably correct or uncertain? Do you consent to finger puncture of your child for blood sampling?

Random blood samplings were made from 324 pre school children (2 to 6 years old) whose parents had consented to it From the 7 years old school children the first 100 samples were randomly collected later an additional number (76) were taken from children who had received 3 doses of vaccine only

Altogether 500 samples were investigated for antibody content All were tested for type 1 and type 3 poliovirus antibodies Only 104 samples were tested for type 2 antibodies

RESULTS

Responses to Questionnaires

Almost 100 per cent of the questionnaires were answered and returned Ninety seven per cent claimed that their child had been immunized to poliomyelitis 2 per cent were reported as unvaccinated and 1 per cent did not remember whether or not the child was vaccinated Eighty nine per cent claimed that their answers were correct (or probably correct) while the rest i e 11 per cent were uncertain Ninety eight per cent consented to blood sampling

Results of Antibody Determinations Antibody Levels in Pre School Children in Relation to Their Year of Birth

The serologically tested pre school children were arranged according to year of birth and vaccination history (Table I) The data from the children whose vaccination data had been claimed to be correct or probably correct are described in detail Table 1 shows that in general 50 per cent of the children had received the first 2 doses of vaccine in the calendar year following that of birth while the majority of the others received them one year later

The distribution of antibody levels in the age groups 2 to 6 years are illustrated by immunologic profiles in Fig 1 Only antibody levels against poliovirus type 1 and 3 are shown The geometric mean titres in the different age groups are shown in Fig 2 The type 2 antibody levels are here also included It can be seen that the antibody distribution appears to be fairly similar in the groups tested also the mean levels do not diverge to any noticeable extent Generally slightly

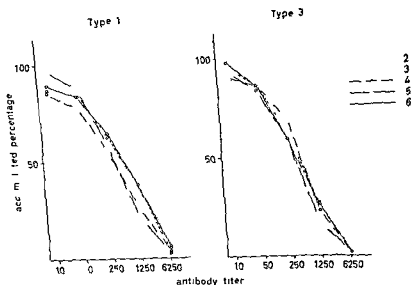


Fig 1

Immunologic profiles i e accumulated titre distribution of different age groups of pre school children The vertical axis gives the accumulated percentage i e the percentage of children attaining or surpassing indicated titre The horizontal axis gives the antibody titre expressed as the reciprocal of the last neutralizing serum dilution The different age groups are indicated in the figure

lower mean titres in the 4 to 5 years old children can be noticed however

Sero-Negative Samples

Among the pre school children studied 9 per cent were found to be sero negative to type 1 and 6 per cent to type 3 in the lowest dilution tested i e a dilution of 1 10 The results are shown at the bottom of Fig 2

Antibody Levels in Relation to Information about Vaccination History

The antibody data were further analysed according to the year of the first injection and the number of injections received The results are illustrated by immunologic profiles in Figs 3 and 4 and by mean titres in Fig 5 Fig 3 shows the antibody distribution after 4 injections The left part of the figure represents type 1 antibody levels and the right part the type 3 levels

It can be noticed that the type 1 titres in the children vaccinated for the first time in the years 1960 and 1961 generally are lower than titres in those vaccinated later The type 1 titres of children vaccinated between 1962 to 1964 show similar distributions however

The type 3 antibody profiles do not display any noticeable difference between the children vaccinated during the years 1960 to 1964

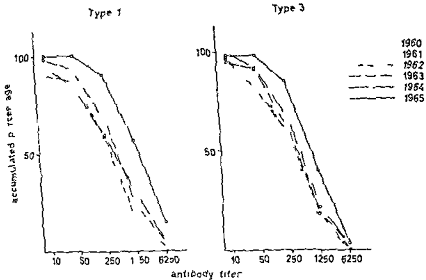


Fig 3

Immunologic piofile of children having received 3 injections of poliovirus vaccine The year when the two first injections were administered is indicated in the figure Other symbols as in Fig 1

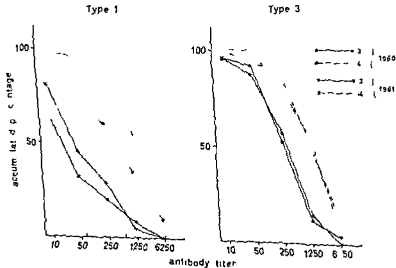


Fig 4

Immunologic profiles" of children having received 3 and 4 doses of vaccine beginning in 1960 and 1961 Symbols as in Fig 1

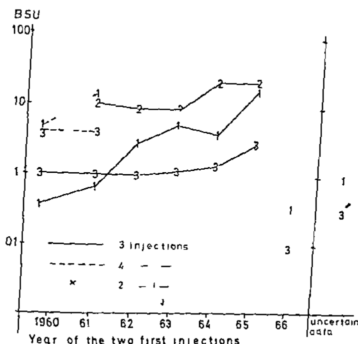


Fig 5

G ometric mean titres (B S U) of serum samples in relation to number of injections and time of the first two injections Symbols as in Fig 2

children whose data were recorded as uncertain or whose vaccinations were incomplete (*i e* less than 3 injections in 1965 or earlier)

Generally high type 2 mean titres can be observed In the children inoculated twice within the last year low titre levels against all three types were generally found The group in which records were uncertain also displayed means below the general levels

Vaccination Record of Sero Negative Children

Fourteen children out of the 500 tested were found to be sero negative to both types of virus investigated With one exception they were reported as unvaccinated (7) not fully vaccinated (3) or the parents were uncertain of the number of injections given (3)

DISCUSSION

Answers to Questionnaires

Ninety seven per cent of the children questioned were reported to be vaccinated against poliomyelitis Since 1960 all children have been offered vaccination at the Well baby clinics Over ninety per cent of Swedish children are regularly brought to these clinics during their first years of life The high frequency of vaccinated children thus

affirms that the system of enrolment for poliovirus vaccination is satisfactory.

Eighty nine per cent of the parents claimed that the information given was correct. This was a surprisingly high figure. No official registration of the names of the individual vaccinees is undertaken. However, every mother is presented with a health card for her baby at the maternity hospital and she is supposed to register all vaccinations on this card. She may also obtain some information from the Well baby clinic. Still, the data may not be as correct as it would be under controlled experimental conditions. The vaccination data (Table 1) of the various age groups follow a very similar pattern, which favours the assumption that the reported data in general may be correct.

Antibody Patterns in Relation to Age

The antibody titre distribution in the age groups 2 to 6 years were found to be almost congruent. This similarity in itself must be regarded as satisfactory. Thus the vaccination programme followed in Sweden apparently has succeeded in inducing an even level of protection in the investigated age groups at the time of this sampling.

It must be stressed that no cases of poliomyelitis have been reported from the area of investigation since 1960 nor has any poliovirus been isolated (3, 4). In the country as a whole an epidemic with 50 paralytic cases occurred in Gothenburg in 1961. Since that time none or 1–2 cases ("imported ones") per year have been registered. Thus the influence of natural infection on any of the results presented here can be disregarded.

Antibody Patterns in Relation to Year of Primary Vaccination

The discrepancy between type 1 and type 3 antibody levels in relation to the date of vaccination *is noteworthy*. The dissimilarity concerns only children receiving their primary vaccination in 1960 and 1961, however. Thereafter the type 1 and type 3 antibody titres follow a very similar pattern. The immunogenic potency values of the Swedish inactivated vaccines had been tested yearly in triple negative children (2, 7). Fig. 6 shows the yearly means of antibody levels in triple negative children tested 2 weeks after 2 injections of inactivated vaccine. According to these tests, titre levels to type 1 were generally found to be lower in the years 1957 to 1961 than in the following years. This change of immunogenicity has been analysed in a previous paper (2) and was found to be coincidental with a change of the seed strain used for vaccine production from the type 1 Stockholm 53 strain to the Brunenders strain. It can also be noticed in the figure that since 1962 the type 1 levels have been similar to the high type 2 and type 3 post vaccination antibody levels which had been reached since 1958 and

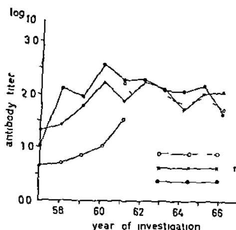


Fig 6

Results of potency tests of inactivated vaccine performed in triple negative children The vertical axis gives the mean titres of the children tested the horizontal axis the year of investigation The type 1 mean titres are illustrated by a whole drawn line when the Stockholm type 3 strain was used as the seed strain for the vaccine The dashed line represent the titres to appear when the Brunenders strain was used

1959 respectively Thus the results of the present investigation are in full agreement with the outcome of the vaccine tests This fact also favours a high validity of the vaccination histories reported

Remarks on Duration of Immunity

Of further interest in Fig 3 and Fig 5 are the remarkably similar antibody levels to type 2 and 3 in children vaccinated from 1960 to 1964 and to type 1 from the years 1962 to 1964 Only the vaccinees who had completed their vaccination with a third dose within the last year of this investigation showed higher levels to all types Since the vaccine potency had not varied considerably in the years mentioned above the results of this investigation suggest that the titres remain fairly stable after the first post vaccination fall of titre has occurred during the year following the vaccination Although this phenomenon was observed by *Salk* (6) in a few cases continuously investigated over extended periods of time it has not been supported before by large scale investigation On the contrary the supposition that antibodies induced by immunization with inactivated vaccine should be less persistent than those raised by vaccination with live attenuated virus has been almost generally accepted

The type 2 titres are generally high which is in accordance with all experience in this field Previous studies by the author have indicated that the kinetics of the antibody response to type 2 antigen differs from that of type 1 and 3 (2) The type 2 antibodies appeared to increase in titre for a longer period of time Thus the levels were generally higher in samples collected 7 months after the 2 primary

injections than after 2 weeks a phenomenon never observed in the case of the other two virus types

The results illustrated in Fig 5 do not contradict this observation The general postvaccination titre levels of type 2 antibodies declined later than those of type 1 and 3

The Effect of a Second booster (the Fourth Dose)

An evaluation of the titre levels in children having received 3 or 4 doses (the latter having received a second booster) suggests (Fig 5) that the effect of the late booster is correlated to the vaccinees immunity status prior to the booster

SUMMARY

The poliovirus vaccination history of close to 1100 children mainly e school children was collected in Stockholm in the autumn of 1966 cording to the information given by the parents 97 per cent had en vaccinated Incomplete vaccinations i e less than 3 injections ministered in 1965 or earlier were reported from 3 per cent of these ighty nine per cent claimed that the vaccination history given was orrect

In 324 randomly selected pre school children antibody titrations howed a similar titre distribution in all pre school age groups A total f 9 per cent was sero negative to poliovirus type 1 in the lowest serum ilution tested i e 1 10 The corresponding figure for type 2 and type 3 was 1 and 6 per cent respectively

Analysis of titres in relation to the date of vaccination suggested a good correlation between previously routine vaccine potency tests performed in children and levels observed in the presently investigated children The children reported to be primarily vaccinated after 1961 showed demonstrable antibodies of the same magnitude to all three types of poliovirus over 95 per cent had demonstrable antibodies in the dilution 1 10

The results also support the assumption that the antibody levels remain fairly stable after the main post vaccination fall has occurred in the year following the immunization

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EXPERIMENTAL STAPHYLOCOCCAL ARTHRITIS IN GOLDEN HAMSTERS (MESOCRICETUS AURATUS)

By

LARS LINDBERG

Received 17.x.68

Investigation of the course of the tissue reaction and other features of an infection often requires model experiments in laboratory animals. Search of the literature for experimental staphylococcal arthritis in small laboratory animals, however, fails to reveal more than a few investigations in which such infections have been induced. Several relevant papers on staphylococcal arthritis in the rabbit are available (*Rigdon* 1942, *Frankel et al.* 1943, 1944 and 1958, *Davis & Ring* 1960, *Bardenheier et al.* 1966), but reports concerning such lesion in the *guinea pig*, *albino rat* and *mouse* are not available, possibly because of the difficulty in inducing staphylococcal arthritis in these animals. The *golden hamster* seems not to have been tried in this respect. In certain experiments it is very difficult, sometimes even impossible, to use an animal as large as the rabbit in investigations requiring very large series of animals or in pharmacokinetical studies using expensive substances such as radioactively labelled antibiotics (*Lindberg & Lundberg* 1969). The purpose of this investigation therefore was to devise a method for producing a model infection with *Staphylococcus aureus* in a joint of a laboratory animal smaller than the rabbit.

MATERIAL AND METHODS

Choice of Bacterial Strain

Staphylococcus aureus was used. As the pathogenicity can be expected to vary from strain to strain, eight strains with positive coagulase reaction against guinea pig plasma at 37° C were selected for examination of their pathogenicity for the guinea pig and the golden hamster. The phage types are given in Table 1. Suspensions of each strain were prepared in the following way.

From the blood agar dish where the strain had been grown, one platinum loopful of the culture was transferred to a test tube containing 4.5 cc of broth and thoroughly homogenized. The broth consisted of: Bacto beef extract 0.3 per cent, Bacto pepton 1 per cent, NaCl 0.3 per cent, $Na_2HPO_4 \cdot 2(H_2O)$ 0.2 per cent, distilled water 98 per cent. The pH of the medium was 7.6–7.8. The test tube was incubated for eighteen hours at 37° C. The amount of the bacteria in the broth was there-

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This investigation was made possible by a grant from Alfred Österlunds Stiftelse

after standardized photometrically by dilution with physiological saline The number of bacteria was 10^9 per cc as judged by dilution series culture and colony counts

Each bacterial strain was tested by intra articular injection of 0 1 cc of suspension into the left knee of two guinea pigs and by injection of 0 05 cc into the left knee of two golden hamsters The animals were observed daily for four weeks for any general or local reaction A slight swelling of the knee joint during the first 1–5 days was the only reaction found in the guinea pigs and a clear swelling lasting throughout the four weeks indicating active infection was observed in all golden hamsters

TABLE 1

Strain	Phage type	Group
1518	29+	I
1596	non specific 52/52A/80	I
P 3844	29+ (79/6557)	I
4432	3 A	II
5843	7/47/54/75/83A/3945/6557	III
5885	3 A	II
5924	77	III
Wood 46	NT	

As the strains did not differ from one another in pathogenicity the well known strain Wood 46 was chosen for the continued investigation The strain had been kept on blood agar dishes at + 4 C and had been subcultured once a month when also the phage type and antibiogram had ben checked

Animal Experiment

For this investigation seventeen golden hamsters weighing about *100 grams* were used 0 05 cc of the bacterial suspension was injected into the left knee of each golden hamster The animals were sacrified at predetermined intervals (Table 2) The infected knee joint was removed fixed in 10 per cent formalin decalcified in formic acid embedded in paraffin Histological sections 7 microns thick were cut and stained with haematoxylin eosin or according to van Gieson

TABLE 2

Number of animals	Killed after
3 + 1S	3 days
4	1 week
3	2 weeks
2	4 weeks
2	6 weeks
2	8 weeks

S = succumbed during experiment

RESULTS

Three days Owing to postmortem autolysis the animal that died spontaneously was excluded from the analysis *In two of the other animals* the joints showed moderately advanced arthritis (Fig 1) *The joint cavity* contained inflammatory cells The synovial membrane was masked by inflammatory changes The joint capsule *and the extra articular tissue* contained inflammatory exudate with numerous polynucleated cells



Fig 1

Hamster knee three days after injection of *Staphylococcus aureus* Incipient abscess formation dorsally in the knee fold and in the suprapatellar recess Dorsally the muscles show infiltration of inflammatory cells van Gieson 115 X



Fig 2

Hamster knee three days after injection of *Staphylococcus aureus* Infection has progressed more rapidly than in the joint in Fig 1 Joint cartilage and bone trabeculae in both epiphyses are partly destroyed van Gieson 115 X

This exudate extended down along the muscles of the lower leg The joint cartilage the epiphyseal lines and the bone marrow of both the tibia and the femur were of normal appearance The nuclei in some of the osteocytes in the bone near the joint were pycnotic

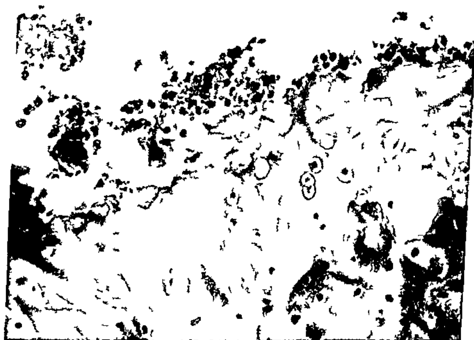


Fig 3
Destruction of joint cartilage three days after injection of *Staphylo ccus aureus* van Gie on 400 ×



F g 4
Ham ter knee one week after injecti n f *Staphyl c us aureus* l rge al e e in ventral and d rsal part of joint Fem ral epiphysis partly destro ed centrally van Gie n 115 ×



Fig 5
Hamster knee two weeks after injection of *Staphylococcus aureus* van Gieson 115 ×

In the joint of the remaining animal the infection was more advanced (Fig 2) The joint cavity and capsule as well as the periarticular tissue were penetrated by pus The muscular interspaces in the thigh contained inflammatory cells The joint cartilages were partly eroded (Fig 3) Necrotic cells were seen in the epiphyseal lines and in several of the bone trabeculae of both epiphyses The entire marrow of the femoral epiphysis was necrotic

One week In three of the animals the infection was more advanced than after three days and the joint cavity was distended by a large amount of pus (Fig 4) All soft tissues in and around the joint contained numerous polynucleated leucocytes The articular cartilages were partly destroyed especially on the ventral aspect of the femoral epiphysis Smaller parts of the epiphyseal lines were necrotic Abscesses were seen in two of the tibial epiphyses In several osteocytes in trabeculae near the joints the nuclei were pycnotic

In the remaining animal the gross anatomical topography was un



Fig 6

Complete destruction of hamster knee two weeks after injection of *Staphylococcus aureus* van Gieson 11 5 ×

changed but the whole joint including the cartilage bone marrow of both the femoral and the tibial epiphyses and metaphyses were totally necrotic Small scattered groups of polynucleated leucocytes were seen

Two weeks In one animal the picture resembled that seen after one week but the amount of pus in the joint cavity had decreased as had the infiltration of inflammatory cells in the periarticular tissues The ventral part of the femoral epiphysis was destroyed (Fig 5)

In the remaining two animals the joints were totally destroyed Only remnants of the epiphysis and the epiphyseal lines could be recognized (Fig 6) The region between the bone ends was occupied by a granulation tissue rich in cells with scattered polynucleated leucocytes partly aggregated to small abscesses

Four weeks In one animal the infection was regressing in that only some parts of the joint capsule and periarticular tissue contained inflammatory cells The joint cartilage ventrally on the femoral epiphysis was eroded and in other areas it was necrotic The epiphyseal line the bone marrow and the bone tissue were of normal appearance



Fig 7

Hamster knee six weeks after injection of *Staphylococcus aureus* Infection healed after partial destruction of the femoral epiphysis van Gie on 11 5 ×

In the remaining animal the picture was the same as in the animal illustrated in Fig 5

Six weeks In both animals the infection appeared to have healed and no pus or inflammatory cells was seen

In one of the animals the infection had healed without demonstrable sequelae In the other the joint cartilage of the femur was partially eroded and there the joint capsule had become firmly attached to the bone (Fig 7) In other areas the joint cartilage was necrotic and contained no stainable cells but the morphology of the joint was otherwise preserved

Eight weeks In both of the animals the infection had healed with complete destruction of the joint

In one animal the joint was destroyed and the area between the bone ends was full of a thick fibrous tissue (Fig 8) In the other animal the subsynovial connective tissue was thickened and the cartilage was partly necrotic and partly eroded with the result that the sub



Fig 6

Complete destruction of hamster knee two weeks after injection of *Staphylococcus aureus* van Gieson 11.5 ×

changed but the whole joint including the cartilage bone marrow of both the femoral and the tibial epiphyses and metaphyses were totally necrotic Small scattered groups of polynucleated leucocytes were seen

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sumption also strengthened by personal unpublished and unsuccessful experiments

In a search for another laboratory animal suitable for this kind of model infection the mouse proved too small to allow satisfactory intra articular infection and it therefore seemed reasonable to try the golden hamster which has apparently not been used before for this purpose

Results

Purulent arthritis developed in most of the animals (in 14 or 15 of 17) It reached a maximum after one week when large amounts of pus were seen in and around the joint Signs of incipient regression appeared after about four weeks and after 6–8 weeks no certain signs of infection were any longer demonstrable

The final picture varied with the severity of the arthritis from complete or partial recovery (Fig 7) to complete (Fig 8) destruction of the joint

Only one of the seventeen animals succumbed during the experiment In view of the high frequency of deaths otherwise claimed when the rabbit has been used for infections of bone and joints (*Lindberg* 1967) this low loss in the present investigation underlines the usefulness of the golden hamster

Judging from the observations set forth above the golden hamster is a suitable animal for infections of the joints with *Staphylococcus aureus*

SUMMARY

A method is described by which it is possible to induce infectious arthritis in golden hamsters by intra articular injection of *Staphylococcus aureus*

All animals displayed purulent arthritis Signs of healing were seen after 4 weeks After 6–8 weeks the infection was healed with extensive destruction of the joints as end result

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THE DISTRIBUTION OF TRITIUM LABELLED DIHYDROSTREPTOMYCIN AND TETRACYCLINE IN STAPHYLOCOCCAL ARTHRITIS

An Autoradiographic Study of Golden Hamsters and Rabbits

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Antibiotics are the most important therapeutics used in the treatment of bacterial arthritis Such therapy however often fails to ward off partial or complete destruction of affected joints It appears that the substance given does not reach or does not affect the bacteria in certain parts of the focus

The purpose of the present investigation was to assess the distribution of dihydrostreptomycin and tetracycline in an infected joint to find out whether any parts of the infected joint are not reached by these antibiotics

In a contemplated series of studies other antibiotics will also be tried The distribution of antibiotics in various body tissues is conventionally assessed by bacteriological determination of the amount of antibiotic in biopsy specimens This method however is relatively crude and does not take into account the fact that different tissues in the biopsy specimen are intermingled and that the pieces of tissue removed often are contaminated with blood and tissue fluid containing antibiotics in high concentration In this investigation a method is used by which it is possible to avoid the above mentioned sources of error of the antibiotic in the various tissues of the infected joint The investigation was carried out on animals with experimental arthritis using an autoradiographic technique described by *Ullberg* (1954) and *André* (1956) which allows location of water soluble substances Since the route of administration of these substances is of interest also clinically the distribution of the antibiotics in the joint was studied both after parenteral and intra articular administration

MATERIAL AND METHODS

Use was made of tritium labelled dihydrostreptomycin sulphate with a specific radioactivity of 29.6 mCi/mg and a radiochemical purity of more than 90 per cent and tritium labelled tetracycline with a specific radioactivity of 41.8 mCi/mg and a radiochemical purity of more than 97 per cent[1]

For the sake of economy the distribution of the antibiotics after parenteral injection was studied in golden hamsters while for technical reasons the distribution after intra articular injection of the substances was studied in rabbits

In the study of the distribution after *parenteral injection* staphylococcal arthritis was produced in golden hamsters by a method described by *Lindberg* (1969) Forty animals weighing about 100 g received an injection of 0.05 ml of an 18 hour broth culture of *Staphylococcus aureus* strain Wood 46 containing about 10^9 colony forming units per ml Two groups of animals with 20 animals in each were used One group received dihydrostreptomycin the other tetracycline Among the animals in the dihydrostreptomycin group 10 were given an injection of the drug 2 days after infection with the bacteria and 10 animals received it 2 weeks after infection with the bacteria The dose consisted of 0.25 ml of dihydrostreptomycin sulphate containing 0.78 mg of dihydrostreptomycin sulphate corresponding to 23 mCi/animal In the tetracycline group the animals received an injection of 0.25 ml of tetracycline solution containing 0.33 mg of tetracycline corresponding to 13.8 mCi/animal 2 days after the infection with the bacteria and the remaining 10 animals a corresponding injection 2 weeks after the bacterial injection All antibiotics were injected intramuscularly under the left scapula Two animals in each of the four subgroups were killed 15 minutes 30 minutes 1 hour 3 hours and 6 hours respectively after the injection of the antibiotic

In the study of the distribution of antibiotics *injected intra articularly* arthritis was produced by injection of 0.2 ml of the same bacterial suspension (see above) into the shoulder joints of 20 rabbits weighing about 2 kg Dihydrostreptomycin sulphate was used in one series tetracycline in the other either drug being injected intra articularly into 5 animals from each group 2 days after the injection of the bacteria (acute arthritis) the remaining 5 animals in each group received it 3 weeks after (destructive arthritis) the injection of bacteria Each joint received an injection of 0.2 ml containing 0.1 mg of dihydrostreptomycin sulphate corresponding to 300 µCi or 0.007 mg of tetracycline corresponding to 300 µCi The animals were killed at the same intervals as those used for the experiment with golden hamsters with the exception that none was killed at the 30 minutes interval

As soon as the animals had been sacrificed the infected joints were removed and frozen in a mixture of hexane and carbon dioxide snow The knee joints were freeze dried for a week embedded in paraffin and tape sectioned (*André* 1956) The shoulder joints were tape sectioned in −15 C according to *Ullberg* (1954) The tape mounted sections were fastened to Ilford G 5 autoradiographic plates After exposure the strip with the adherent sections and the autoradiographic film were separated the autoradiogram developed and fixed finally the sections were stained with haematoxylin eosin and mounted The autoradiogram and the histological sections were afterwards compared by placing the section over the autoradiogram

RESULTS

Parenteral Administration

Neither in the group that received dihydrostreptomycin nor in the one that received tetracycline 2 days after injection of the bacterial suspension did the distribution pattern of the antibiotics differ with certainty from that found when the antibiotic was not given until 2 weeks after the bacterial injection

[1] The tritium labelled dihydrostreptomycin and tetracyclin were obtained from Avd Ingen f r Isotoptjanst AB Atomenergi Studsvik Sweden Purified chlortetracyclin and streptomycin sulphate for preparation of the radio active substances was courteously supplied by AB Astra Sodertalje Sweden

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THE DISTRIBUTION OF TRITIUM LABELLED DIHYDROSTREPTOMYCIN AND TETRACYCLINE IN STAPHYLOCOCCAL ARTHRITIS

An Autoradiographic Study of Golden Hamsters and Rabbits

By

LARS LINDBERG and BO LINDBERG

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Antibiotics are the most important therapeutics used in the treatment of bacterial arthritis. Such therapy, however, often fails to ward off partial or complete destruction of affected joints. It appears that the substance given does not reach, or does not affect, the bacteria in certain parts of the focus.

The purpose of the present investigation was to assess the distribution of dihydrostreptomycin and tetracycline in an infected joint to find out whether any parts of the infected joint are not reached by these antibiotics.

In a contemplated series of studies other antibiotics will also be tried. The distribution of antibiotics in various body tissues is conventionally assessed by bacteriological determination of the amount of antibiotic in biopsy specimens. This method, however, is relatively crude and does not take into account the fact that different tissues in the biopsy specimen are intermingled and that the pieces of tissue removed often are contaminated with blood and tissue fluid containing antibiotics in high concentration. In this investigation a method is used by which it is possible to avoid the above mentioned sources of error of the antibiotic in the various tissues of the infected joint. The investigation was carried out on animals with experimental arthritis, using an autoradiographic technique described by *Ullberg* (1954) and *André* (1956) which allows location of water soluble substances. Since the route of administration of these substances is of interest also clinically, the distribution of the antibiotics in the joint was studied both after parenteral and intra-articular administration.

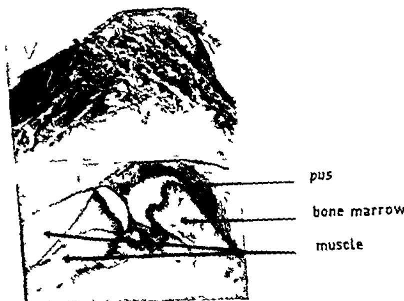


Fig 5

Histological section and corresponding autoradiogram of knee infected two days previously The animal was killed 1 hour after the injection of tritium labelled dihydrostreptomycin Dihydrostreptomycin is still concentrated in the pus filled joint Haematoxylin Magnification × 8

larly injected tetracycline activity was demonstrable only locally in subchondral bone

DISCUSSION

In an planned investigation of a series of antibiotics with the procedure described above dihydrostreptomycin and tetracycline were selected first because they are easy to label with tritium and are relatively cheap and second because they are metabolized to only a small extent in the body (*Andre* 1956 *Lindberg* 1967)

Dihydrostreptomycin is no longer used in human medicine because of its occasional side-effects on the auditory nerve It was however used in the present investigation since it apparently has the same pharmacokinetic and antibacterial properties as streptomycin and since it is much easier to label with tritium than streptomycin

Successful autoradiography with the technique used here requires first prevention of redistribution of the water soluble radioactive substance and second histological sections of *undecalcified bones* With the degrees of microscopical magnification used in the present investigation no redistribution of either antibiotic was observed The possibilities of using higher magnification of the tissue is limited by the fact that the material contains undecalcified bone which is difficult to section

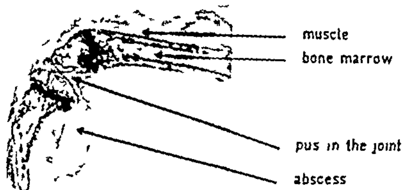


Fig 3

Autoradiogram of knee infected 2 days previously The animal was killed 15 minutes after injection of tritium labelled dihydrostreptomycin Dihydrostreptomycin has not yet entered one abscess in the suprapatellar recess The bad quality of the autoradiogram is due to difficulties in sectioning the non decalcified bone tissue Magnification × 10



Fig 4

Autoradiogram of knee infected 2 days previously The animal was killed 20 minutes after injection of tritium labelled tetracycline Tetracycline has not yet entered an abscess dorsally in knee Magnification × 8

acute arthritis until at least 3 hours after the injection The effect of administration by this route appeared to be confined practically to the joint cavity since no activity of either substance was demonstrable periarticularly (Fig 8) In more severely destroyed joints the distribution both of dihydrostreptomycin and of tetracycline during this period was most complete in abscesses communicating with the site of injection but neither antibiotic had penetrated into abscesses not in open communication with the joint nor had they diffused beyond the borders of the affected joints (Fig 9) At this stage in contrast to findings in acute arthritis the streptomycin persisted 6 hours in infected communicating areas 3 and 6 hours after the injection of the intra articu

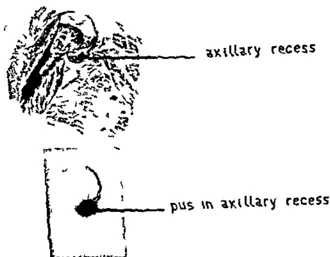


Fig 8

Histol gical section and corresp nding autoradiogram of shoulder joint infected 9 days previously The animal was killed 3 hours after the intra arti ular injection of tritium labelled dihydrostreptomycin The antibiotic is *confined* to the cavity and cartila e of the joint Haematoxylin Magnification × 1

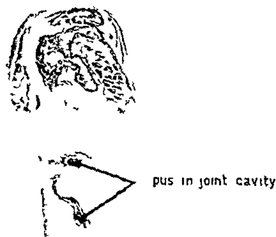


Fig 9

istological section and corresponding autoradiogram of houlder joint infected weeks previously The animal w s killed 1 hour after the intra articular inje tion tritium labelled dihydrostreptomycin As in acute arthritis the antibiotic is confined to the joint cavity Haematoxylin Magnification × 1

The present investigation would not allow any conclusions about differences in absolute concentration values between *dihydrostreptomycin* and tetracycline because these two antibiotics differ considerably *in specific radioactivity* Only conclusions about the relative distribu*tion patterns can be drawn*

The difference between the autoradiographic distribution of dihydrostreptomycin and that of tetracycline in healthy joint tissue *following parenteral administration* described by *André* (1956) was seen also in the present study Dihydrostreptomycin accumulates in mesenchymal *tissues such as cartilage* periosteum connective tissue etc Tetracycline tends to accumulate in bone tissue but is otherwise diffusely distributed In a few abscesses produced by intramuscular injections of *Corynebacterium pyogenes André* also found the two antibiotics to penetrate the lesions Here dihydrostreptomycin was even observed in a higher *concentration than that in the surrounding tissue However* the material was too small to permit any general conclusions In an investigation with the same technique *Lindberg* (1967) found that the concentration of dihydrostreptomycin was relatively high in tuberculous osteomyelitic abscesses

Observations made in the present investigation show that very high concentrations of dihydrostreptomycin can be achieved in pus in joints *infected with staphylococci* The accumulation of dihydrostreptomycin in pus occurs rapidly Already after 15 30 minutes the concentration of dihydrostreptomycin in pus filled joints is much higher than in sur*rounding* soft tissues (*Fig 1*)

Tetracycline appears to diffuse into the tissues at the same rate as dihydrostreptomycin after 15 30 minutes tetracycline was found in all tissues except in the central part of one abscess (Fig 4) However the distribution pattern in the tissues is quite different the concentration *of tetracycline in the abscesses and in tissue rich* in inflammatory cells was roughly the same as in surrounding tissue an observation also made by *Andre* in soft tissue abscesses

After 3 hours the concentration of both the dihydrostreptomycin and tetracycline in all soft tissues had begun to fall because of elimination *of the substance from the body* and after 6 hours the autoradiogram showed no signs of antibiotic except in calcified bone where the con centration of tetracycline remained unchanged

The fact that the concentration of dihydrostreptomycin *was high in* the contents of the abscess while that of tetracycline was not higher than the concentration in surrounding tissue can perhaps be explained on the assumption that large amounts of dihydrostreptomycin but not of tetracycline are reversible bound to certain components of the con tents of the abscess (*Lindberg* 1967) The nature of the bond is not clear but it is probably a protein bond The amount of dihydrostreptomycin bound would than be able to act as a depot from which anti bacterially active dihydrostreptomycin is supplied *when the concen*

tration in the serum and surrounding tissues falls owing to excretion of the antibiotic from the body

Two factors which have been considered in the evaluation of the autoradiographic results of *intra articularly injected antibiotic* are the extent to which it spread in the infected joint and the time the antibiotic persists there after such administration It is obvious that in acute arthritis the joint cavity is completely filled with antibiotic whether dihydrostreptomycin or tetracycline is used This distribution can be explained on physical grounds and the antibiotic is evenly distributed in the entire joint cavity In the animals in which arthritis had persisted for 3 weeks the antibiotics was distributed according to the same mechanism At this stage of destruction and repair some encapsulated foci however appear inaccessible to intra articularly injected antibiotic No antibiotic was seen periarticularly Investigations with the same autoradiographic technique in connection with measurement of the disappearance rate of I^{131} Hippuran and $Na^{22}Cl$ from the shoulder joint of the rabbit showed that already after 15 minutes the same volume corresponding to 10 μCi had spread periarticularly (*Lundberg* 1969) The limited penetrability of the antibiotics studied here is probably due to the largeness of their molecules The blackened areas over the abscesses even 6 hours after the injection of dihydrostreptomycin indicate the binding of this antibiotic in the abscess The greatest advantage of administration by the intra articular route is probably that it is possible to achieve a high local concentration of antibiotic The clinical application of intra articular injection of these antibiotics in arthritis seems however limited especially in the treatment of chronic forms of the disease Our results suggest that simultaneous parenteral administration is necessary if all affected tissues are to be reached by the antibiotic

SUMMARY

The distribution of dihydrostreptomycin (for technical reasons used instead of streptomycin) and tetracycline in experimental staphylococcal arthritis has been studied with autoradiographic technique After intramuscular injection both antibiotics are found to penetrate well into the infected tissues even into abscesses Dihydrostreptomycin is found to be concentrated in pus compared to normal soft tissues whereas tetracycline is found to be evenly distributed among all soft tissues The distribution after intra articular injection of the antibiotics is complete but strictly limited to the joint cavity and communicating infectious foci until 3 hours after the injection

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SUBTYPING OF HUMAN HAPTOGLOBINS WITH PRECIPITATING ANTISERA I

By

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Three common types of human haptoglobin (Hp 1-1 Hp 1-2 and Hp 2 2) can be distinguished on the basis of starch gel electrophoresis patterns (1) *Connell Dixon & Smithies* subjected purified haptoglobin to reductive cleavage and electrophoresis in acid starch gel containing mercapto ethanol and 8 M urea (2) Using this method they found two kinds of polypeptide chain α and β Three types of α chain were distinguishable Hp 2α Hp 1Fα and 1Sα Family studies (3) indicated that these are the expressions of three alleles Hp^{1} Hp^{2} and Hp at the Hpα locus The β chain is the same in all the common phenotypes (4) Thus it is possible to divide the Hp 1-1 phenotype into three subtypes 1F 1F 1F 1S and 1S-1S Chemical studies of α chains made by *Smithies et al* (5) and *Black & Dixon* (6) revealed that 1Fα and 1Sα differ only in one amino acid (lysine in Hp 1Fα is replaced by glutamine in Hp 1Sα) and that the polypeptide Hp 2α is essentially Hp 1Fα plus Hp 1Sα

The subtyping of haptoglobins by electrophoresis of purified haptoglobins in urea mercapto ethanol gels is laborious This reduces the practical usefulness of these characteristics as markers in genetic studies and forensic serology An immunological method for subtyping would be simple and should therefore make haptoglobin subtyping more useful

So far a distinction between Hp 1F-1F and Hp 1S-1S by serological methods has not succeeded However there are several instances where the products of allelic genes have been successfully differentiated using precipitating hetero antisera for example Lp (7) Gm (8) Gc (9) Hb (10) Therefore I made a further attempt to produce antisera specific for Hp 1F and Hp 1S which would allow Hp 1-1 sera to be subtyped by gel double diffusion

I purified 1F 1F and 1S-1S haptoglobins by the following method The sera were dialysed against 0 01 M Na acetate buffer pH 4 7 and applied to a column of DEAE Sephadex equilibrated with the same buffer The column was washed with 0 01 M Na acetate-0 03 M NaCl

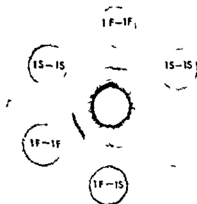


Fig 1

Double diffusion in agar gel The absorbed anti Hp 1F serum in the center tested against sera belonging to different haptoglobin subtypes

buffer and eluted with a buffer containing 0 01 M Na acetate 0 14 M NaCl The haptoglobin rich eluate was concentrated by ultrafiltration and dialysed against Tris (0 1 M) HCl buffer pH 8 Gelfiltration was performed in a 2 × 90 cm column of Sephadex G 200 (Pharmacia Sweden) with the same buffer and a flow rate of 6 ml/hour The fractions were tested immunologically by gel double diffusion with antisera against haptoglobin and normal human serum (anti NHS Behringwerke)

The fractions containing mainly haptoglobin were then dialysed against 1 per cent glycine and applied to an Electrofocusing column (LKB Sweden) 15 mg protein/run The column was run for 72 hours at 500 V in a pH gradient from 3 to 6 Fractions of 2 5 ml were collected and the protein content was determined by measuring the optical density The fractions were tested by gel double diffusion as before The fractions giving only one precipitin line against anti Hp and anti NHS antisera were pooled dialysed against phosphate buffer and stored at —20° C Sera were subtyped according to *Smithies (3)* Rabbits and sheep were immunized subcutaneously with 0 5 and 1 mg/injection respectively of the pure haptoglobin preparations emulsified in complete Freund s adjuvant (Difco) The injections were given weekly for one month and then every third week for at least 4 months The anti Hp sera obtained here called anti Hp 1F and anti Hp 1S usually contained minute amounts of antibody against other serum proteins These contaminating antibodies were removed by absorption with anhaptoglobinaemic serum (5 mg of lyophilized serum to 1 ml of antiserum) When several human sera *belonging to different* Hp groups were tested against the absorbed *antisera* no spur formation could be detected The anti *Hp 1F serum* was further absorbed with different amounts of 1S–1S serum and the anti Hp 1S similarly with

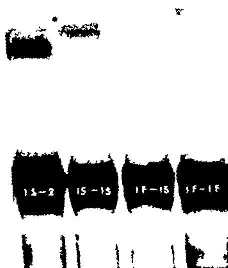


Fig 2

Starch gel electrophoresis patterns of some common haptoglobin subtypes

1F 1F serum Extensive absorption removed all reactivity against haptoglobin from the antisera but less thorough absorption resulted in an anti Hp 1F serum that reacted with Hp 1F–1F Hp 1F–1S Hp 1–2 and Hp 2 2 sera but not with Hp 1S–1S sera (Fig 1) The absorbed anti Hp 1S serum reacted with all but 1F–1F sera

So far I have analysed 20 1S–1S sera 10 1F–1F sera 20 1F–1S sera 15 1 2 sera and 60 2–2 sera and all the results agree with those obtained by subtyping by electrophoresis in urea mercaptoethanol gels (Fig 2)

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A PAPER DISC TECHNIQUE FOR STUDYING ANTIBACTERIAL SYNERGISM

By

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In routine sensitivity testing of bacteria by means of the paper disc method we occasionally observed that *Proteus* strains showed an inhibition zone of an irregular appearance at the colistin disc. This "windscreen wiper" zone, the principal appearance of which is shown in Fig. 1, was only seen in the presence of a sulphonamide disc placed at a limited distance from the colistin disc. Our observation confirming some earlier reports of a positive interaction between sulphonamide and colistin (4) suggested to us a true synergistic effect of these drugs on *Proteus* and inspired a closer study.

Evidently paper disc methods can be used to study synergism. In fact such methods have been proposed (1, 3). In a preliminary sensitivity test the inhibition zone diameters for the different antibiotics are recorded. A second plate is then inoculated with the same strain and two discs, each containing one of the possibly synergistic drugs, are placed on the agar surface at a distance from each other corresponding to half the sum of the previously observed zone diameters. If the antibacterial drugs have a synergistic action on the strain in question, an increased effect is observed in the tangential region of the two inhibition zones.

A prerequisite for this method is obviously that at least one—and preferably both—of the drugs have some inhibitory effect on the strain. It has also been stated (1) that "a drug must have some action alone against a micro-organism in order to be an effective member of a synergistic pair". *Proteus*, however, is a species that is generally resistant to colistin (5) and often also to sulphonamides, and consequently we had to modify the technique to suit even such conditions.

MATERIAL

The strains were isolated from clinical specimens sent to the laboratory for bacteriological diagnosis. In all, 19 strains of *Proteus mirabilis*, six of *Proteus vulgaris* and seven of each of *Staphylococcus aureus*, *E. coli* and *Enterococcus* were tested.

Paper discs prepared as described by *Ericsson Högman & Wickman* (2) were obtained from Karolinska Sjukhuset Stockholm The discs contained 2.4 *mg* of sulphonamide and 30 *mcg* of colistin respectively

All tests were performed on agar plates prepared from Oxoid Blood Agar Base with the addition of 0.1 per cent glucose

RESULTS

In the first part of the study we used the disc arrangement shown in Fig. 1 On an inoculated agar plate colistin discs were placed around a central sulphonamide disc at varying distances from the latter The distances ranged from 7.5 to 40 mm A gauge drawn on white paper and placed under the transparent plate was used to simplify the exact placing of the discs The plates were left for two hours at room temperature for prediffusion and were then incubated at 37° C over night

By means of this spiral plate method the 25 *Proteus* strains were investigated All the strains had previously been found to be resistant to colistin and 12 of them also to sulphonamide 24 of the strains showed a pattern similar to that in Fig. 1 while the remaining strain showed no evidence of synergism At the colistin disc inhibition zones were observed varying in size and form with the distance between the sulphonamide and colistin discs At a certain distance between the discs the typical windscreen wiper zone was seen This distance differed for different strains An imaginary line connecting the outer margins of the inhibition zones formed a circle with the sulphonamide disc as centre When the distance between the discs was too large no inhibition zone was observed

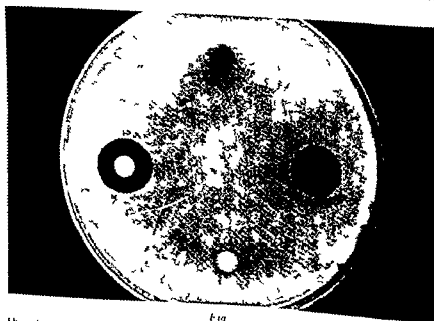
Experience and some preliminary experiments had shown that the antibacterial drugs in the paper discs diffused rather rapidly into the agar when the discs were placed on an agar plate After one hour most of the drug appeared to be in the medium Based upon this observation a disc replacement technique was developed Two colistin and two sulphonamide discs were placed on an inoculated agar plate as shown in Fig. 2 To ensure rapid diffusion the discs were moistened with a loopful of sterile distilled water The plate was left at room temperature for one hour Then the sulphonamide discs were removed and replaced by a colistin disc and another sulphonamide disc respectively The two original colistin discs were replaced by a sulphonamide disc and another colistin disc The plate was left for two hours at room temperature for prediffusion and then incubated at 37 C over night

By this technique the synergism between sulphonamide and colistin could be further established Clear zones of inhibition were observed when a sulphonamide disc had been replaced by a colistin disc and *vice versa* On the other hand no inhibition was obtained when the discs had been replaced by another disc with the same drug in spite of the doubling of the amount of the drug The 12 *Proteus* strains resistant to both colistin and sulphonamide were tested with the replacement tech



Fig 1

A *Proteus mirabilis* strain tested by means of the "spiral plate" method (see text). In the centre a sulphonamide disc and around it at varying distances colistin discs



Fig

[illegible] ment technique ([illegible] text) applied to a *Proteus mirabilis* strain [illegible] A sulphonamide disc replaced by another sulphonamide disc (Right) A colistin [illegible] by a sulphonamide disc. Bottom: A colistin disc replaced by another [illegible] (Left) A sulphonamide disc replaced by a colistin disc

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BRIEF REPORTS

INTERFERENCE FILTERS FOR IMPROVED IMMUNOFLUORESCENCE MICROSCOPY

By *Jørgen Bygaard* and *Werner Olsen*

Exact calculation and production of interference filters with high transmission for use in fluorescence microscopy is possible today thanks to new design methods based on computer technique (5)

In this way a two band interference filter for use as a primary filter for tracing with fluorescein isothiocyanate (FITC) has been developed The main band used for excitation of the fluorochrome gives up to 85 per cent transmission in the 400–495 nm range (blue) With the light transmitted in this band a bright yellow green specific fluorescence can be obtained with an ordinary tungsten lamp (*e g Zeiss low voltage 12 volt 60 watt*) Autofluorescence is negligible using these wavelengths for excitation The second band gives about 1 per cent transmission in a narrow range around 630 nm (red) This band has been added for two purposes namely in order to obtain a highly contrasting background colour for the fluorescence and in order to allow easy localization of fluorescence For most purposes this red band will render any additional phase contrast equipment superfluous

The strongest activation of a fluorochrome is obtained by the wavelengths that are maximally absorbed by the dye (3) The most commonly used fluorescent conjugates are with FITC and have an absorption maximum at 495 nm in the visible part of the light spectrum with an additional medium high plateau around 300 nm in the invisible part of the spectrum The emission maximum of FITC conjugates is at 520 nm (3)

The optimal primary filter should have high transmission at the absorption maximum of the fluorochrome thus giving ample energy for excitation but must of course not overlap the emission maximum For practical purposes it has not so far been possible to apply this theoretical optimum of activation in tracing with FITC because the primary glass filters commonly available have not permitted satisfactory selective filtering The primary filters used in most studies published are the BG 12 filter from Schott & Gen Mainz or similar types of other brands often in combination with a red stopping filter e g type BG 38 This combination will give an ultraviolet blue transmission with its maximum at 400 nm As secondary filters are used yellow orange glass filters opening in the range of 500 nm e g OG 1 or OG 5 (2) The BG 12 primary filters are used with high pressure mercury lamps They will transmit only a small quantity of light at the theoretical optimum for activation of the fluorescence of FITC In addition to being less effective activators of FITC the shorter light wavelengths may give rise to varying degrees of autofluorescence of tissues so that distinction between specific immunofluorescence and autofluorescence may be difficult and sometimes even impossible

The use of an iodine quartz illumination in combination with various filter combinations has been suggested (4 (?) Tomlinson (6) sandwiched two Wratten gelatine filters Nos 32 and 38A giving 66 per cent transmission of 460 nm and 21 per cent transmission at 490 nm Good results were obtained but still the filter combination was characterized as being not ideal because of the low transmission at the wavelengths required for excitation

As pointed out by Nairn There is no doubt that performance of the fluorescence microscope can be improved if optimum filter system are used (3)

Transmission curves of a new primary interference filter designed to meet opti

Received 14 ii 69 *from the Department of Treponematoses Statens Seruminstitut 2300 Copenhagen S and Laboratory for Technical Optics A T I 2800 Kgs Lyngby Denmark*

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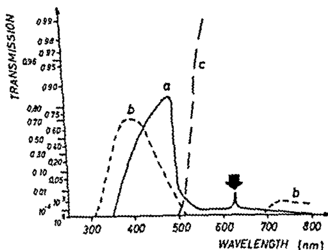


Fig 1

Transmission curves of primary and secondary filters (a) Interference filter arrow indicating contrast band (b) BG 12/3 Schott & Gen (c) OG 1 Schott & Gen

mum requirement are given in Fig 1 With FITC as the tracer a traditional set up for fluorescence microscopy has been compared with the new system In that primary filters BG 38 2.5 + BG 12/3 with the Osram HBO 200 as the light source were compared with the two band interference filter and the Zeiss 60 watt tungsten lamp For both systems a Zeiss photomicroscope with the following equipment was employed condenser Zeiss dark field oil immersion objective Zeiss Apo 40/1.0 oil immersion optovar 1.25 × and eyepieces Zeiss Kpl 8 × giving a 400 × magnification Secondary filters Zeiss 47 50 and 53

The result favoured the interference filter combined with the 60 watt lamp The secondary filter of choice was the Zeiss 50 (515 nm) filter giving the most suitable red transmission of the three secondary filters tested and thus the best background contrast Test objects were frozen sections of liver thyroid muscle and leucocyte smears tracing for antinuclear factors and other autoantibodies in human sera A FITC conjugated anti human gamma globulin was used Sections were mounted in buffered glycerol In addition the fluorescent treponemal antibody (FTA) test was performed successfully with the interference filter system

The main advantages of the interference filter are the bright specific immunofluorescence with a simple light source the high contrast between fluorescence and background tissue and the easy localization of fluorescent areas in the cells or tissue there is also negligible autofluorescence Furthermore a so far unknown richness in details is seen due to both the absence of autofluorescence and the optimal colour contrast

We believe that immunofluorescence with FITC conjugates will be facilitated by this new system and that new fields in searching for weak antigens and antibodies may be opened e.g. in auto immunity and tumour immunity research. The system can be adapted for other fluorochromes also The tungsten lamp may be replaced by a halogen lamp

These light sources are stable thus facilitating microphotometry and need no heating period before use The slightly higher cost of interference filters as compared to ordinary glass filters is more than compensated by the low cost of light source and transformer and the omittance of combined systems e.g. phase fluorescence for orientation

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Acta path microbiol scand 76 146–148 1969

BRIEF REPORTS

INTERFERENCE FILTERS FOR IMPROVED IMMUNOFLUORESCENCE MICROSCOPY

By *Jørgen Rygaard* and *Werner Olsen*

Exact calculation and production of interference filters with high transmission for use in fluorescence microscopy is possible today thanks to new design methods based on computer technique (5).

In this way a two band interference filter for use as a primary filter for tracing with fluorescein isothiocyanate (FITC) has been developed. The main band used for excitation of the fluorochrome gives up to 85 per cent transmission in the 400–495 nm range (blue). With the light transmitted in this band a bright yellow green specific fluorescence can be obtained with an ordinary tungsten lamp (e.g. Zeiss low voltage 12 volt 60 watt). Autofluorescence is negligible using these wavelengths for excitation. The second band gives about 1 per cent transmission in a narrow range around 630 nm (red). This band has been added for two purposes, namely in order to obtain a highly contrasting background colour for the fluorescence, and in order to allow easy localization of fluorescence. For most purposes this red band will render any additional phase-contrast equipment superfluous.

The strongest activation of a fluorochrome is obtained by the wavelengths that are maximally absorbed by the dye (3). The most commonly used fluorescent conjugates are with FITC and have an absorption maximum at 495 nm in the visible part of the light spectrum with an additional medium high plateau around 300 nm in the invisible part of the spectrum. The emission maximum of FITC conjugates is at 520 nm (3).

The optimal primary filter should have a high transmission at the absorption maximum of the fluorochrome, thus giving ample energy for excitation, but must of course not overlap the emission maximum. For practical purposes it has not so far been possible to apply this theoretical optimum of activation in tracing with FITC, because the primary glass filters commonly available have not permitted satisfactory selective filtering. The primary filters used in most studies published are the BG 12 filters from Schott & Gen, Mainz, or similar types of other brands, often in combination with a redstopping filter, e.g. type BG 38. This combination will give an ultraviolet-blue transmission with its maximum at 400 nm. As secondary filters are used yellow-orange glass filters opening in the range of 500 nm, e.g. OG 1 or OG 5 (2). The BG 12 primary filters are used with high pressure mercury lamps. They will transmit only a small quantity of light at the theoretical optimum for activation of the fluorescence of FITC. In addition to being less effective activators of FITC, the shorter light wavelengths may give rise to varying degrees of autofluorescence of tissues, so that distinction between specific immunofluorescence and autofluorescence may be difficult and sometimes even impossible.

The use of an iodine quartz illumination in combination with various filter combinations has been suggested (4, 6). *Tomlinson* (6) sandwiched two Wratten gelatine filters Nos 32 and 39A giving 66 per cent transmission of 460 nm and 21 per cent transmission at 490 nm. Good results were obtained, but still the filter combination was characterized as being not ideal because of the low transmission at the wavelengths required for excitation.

As pointed out by *Nairn*, there is no doubt that performance of the fluorescence microscope can be improved if optimum filter systems are used (3).

Transmission curves of a new primary interference filter designed to meet opti

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Requests for reprints should be addressed to Dr Jørgen Rygaard, Department of Treponematoses, Statens Seruminstitut, 2300 Copenhagen S, Denmark

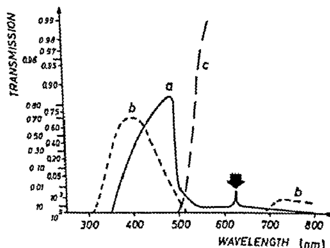


Fig 1

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—3 Fluorescent Protein Tracing 2nd Ed (Ed R C Nairn) F & I Livingstone Ltd Edinburgh and London 1964 p 44 60 & 66–67 —4 Johnson (D & Dollhopf I L. Immunology 1 619 620 1968 —5 Olsen W Unpublished data —6 Tomlinson A H Immunology 13 323 324 1967

Acta path microbiol scand 76 148–149 1969

IDENTIFICATION OF AN ASACCHAROLYTIC *NEISSERIA* STRAIN CAUSING MENINGITIS

By *Kjell Bøvre*

Previous investigations revealed that quantitative genetic transformation can be a valuable tool for the correct arrangement of bacterial strains in a classification system (3) The following demonstrate the practical diagnostic application of this method in the identification of an aberrant *Neisseria* phenotype isolated from a patient with meningitis

Clinical Report

K K a boy born on January 1 1966 was admitted on August 1 1966 to the Pediatric Department Rikshospitalet University of Oslo Upon admission he had pronounced clinical signs of meningeal irritation and his cerebrospinal fluid contained numerous leucocytes Treatment was successfully performed with sulfonamide and penicillin C according to the bacteriological finding

Conventional Bacteriology

The strain B 8152/66 was isolated on a blood agar plate inoculated with cerebrospinal fluid from the patient on the day of admission It appeared as a homogeneous culture of dimethyl oxidase positive Gram negative diplococci with colonial characteristics typical of *Neisseria meningitidis* without pigment production or haemolysis The nitrate reduction test (in serum containing medium) was negative However all colonies examined were also negative in the ordinary tests for saccharolytic activities with 1 per cent glucose maltose and sucrose each incorporated in slants of nutrient agar with 24 per cent ascitic fluid The strain was highly sensitive to sulfonamide penicillin C streptomycin chloramphenicol oxytetracycline and erythromycin as revealed by a plate diffusion test (3)

On the basis of the characters mentioned these differential diagnoses were considered 1) asaccharolytic *N meningitidis* 2) nonpigmented *N flavescens* and 3) nitrate negative *N catarrhalis* The descendants of a single colony provided the material for the following investigation

Genetic Transformation

The wild type of strain B 8152/66 was used as recipient in transformation experiment which generally followed the quantitative procedure described (3) with stock transforming DNA preparations of streptomycin resistant (SR) mutants of *N catarrhalis* 1/910/62 *N flavescens* ATCC 13115 and *N meningitidis* MF all of which had been employed previously (3) In order to provide a tentative species diagnosis in a few days autologous SR DNA had to be omitted at this stage The

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Credit is due to Mr F Saltroe medical student who took part in the initial diagnostic procedure

numbers of streptomycin resistant transformants elicited by the three donors per ml of the same recipient population were as follows N. *catarrhalis* 10 or less N. *flavescens* 1 × 10 and N. *meningitidis* 1.6 × 10³. These results strongly indicated the species diagnosis N. *meningitidis* for the new isolate. For confirmation another quantitative experiment was performed with identical aliquots of the recipient B 8159/66 simultaneously exposed to N. *meningitidis* M6 SR DNA and to the autologous B 8159/66 SR DNA. The interstrain reaction gave rise to 3.8 × 10⁴ transformants per ml and the autologous reaction revealed 6.4 × 10⁴ transformants per ml (0.01 per cent of the recipient count). The resulting ratio of inter strain to intra strain (autologous) transformation is in the order of 0.6, which is compatible with a first degree interrelationship as found between strains of the same species (3). The reciprocal quantitative experiment with N. *meningitidis* M6 as recipient was corroborative.

Further Cultural Tests

After having established the *diagnosis* by transformation, the saccharolytic potential was retested by daily readings for 10 days of cultures at 33 C and 37 C on the carbohydrate media described and also on the same basis medium with 10 per cent of the respective carbohydrates. No positive reaction was observed except for one instance where the 10 per cent maltose ascites agar slant revealed a weak acid reaction after 3 days at 37 C. Following 4 successive probably nonselective subcultures from this slant on blood agar it was finally observed that 9 out of 10 single colonies tested consisted of cells with the glucose+/maltose+/sucrose− pattern typical of N. *meningitidis*. Limited attempts to find a change to typical saccharolytic pattern also in other subculture lines of the same strain, including the streptomycin resistant mutant, all failed. The strain was also asaccharolytic on solid and fluid Mueller-Hinton media (2).

Comments

Glucose−/maltose−/sucrose− strains of N. *meningitidis* were described as early as at the beginning of the century (1). However strain B 8159/66 is the first clinical isolate of this variety which has been shown to be N. *meningitidis* by genetic means. Recently *Jyssum & Jyssum* described mutator induced mutants of this species with a similar defect in carbohydrate metabolism (4).

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Acta path microbiol scand 76 150-160 1969

TRANSACTIONS OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting November 29-30, 1968

Nilseth Ch & Schnürer L. B DISEASES OF THE THYROID IN AN AUTOPSY SERIES FROM MID SWEDEN

Strongly diverging figures on the frequency of primary thyroid malignant tumours in autopsy series have been published In some large materials the figures were as low as 0 1 per cent but in more recent studies in which the thyroid was carefully examined figures as high as 1-3 per cent were reported

Our material was obtained from 500 autopsies performed in 1968 at the Department of Pathology Regionsjukhuset Örebro After weighing and fixation in formalin the thyroid was sliced and several slides including such from all grossly visible changes were studied using van Gieson as routine stain

The average weight of the thyroid was found to be high 32 4 g and it tendend to increase with age High figures were also obtained for the frequency of goiter which was considered as present if the thyroid gland weighed 35 g or more

Chronic lymphoid thyroiditis was found in 12 subacute thyroiditis in 2 cases

Thirty two benign tumours were found

Six primary malignant tumours all carcinomas were found 4 occurred in men and 2 in women Three were fairly well differentiated papillary carcinomas and 3 were small sclerosing rather papillary carcinomas The frequency in our series 1 2 per cent corresponds fairly well to that of recent reports claiming that figures for primary thyroid malignant tumours in such specially studied series are 10 to 20 times higher than the figures obtained from routine materials

Among 115 primary malignant tumours outside the thyroid 14 (12 2 per cent) had thyroid metastases a surprisingly high figure considering the common statement that this condition is seldom encountered

Enerbäck L & Lundvall O LIVER CELL FLUORESCENCE IN PORPHYRIA CUTANEA TARDA (PCT)

The liver of patients with cutaneous hepatic porphyria contains preformed porphyrins exhibiting red fluorescence after fixation with ultraviolet or visible light The properties and distribution of hepatic porphyrins were studied by fluorescence microscopy on aspiration biopsy smears and biopsy sections from 19 patients with latent or manifest PCT

The porphyrins were found to occur in liver cell cytoplasm in freely diffusible form necessitating the use of special techniques for demonstration of the proper localization Freeze drying and formaldehyde gas fixation or fixation in absolute ethanol followed by paraffin embedding gave good results provided that the sections were flattened in the dry state and mounted in water free media If sections were floated onto water most of the fluorescent material was extracted from the sections and the remaining fluorescence was found in liver cell cytoplasm and nuclei or in portal connective tissue

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Air dried smears from fine needle aspiration biopsies contained red fluorescence of varying intensity localized to cytoplasm and nuclei of liver cells Various attempts to avoid the artefactive nuclear fluorescence were unsuccessful Liver cell fluorescence was found in all examined cases of PCT regardless of stage of clinical activity of the disease and in none of 30 control subjects without signs of the disease The extremely simple method of aspiration biopsy should be a useful diagnostic tool in cases of PCT

Hof r P A & Andersson R POST MORTEM FINDINGS IN A CASE OF FAMILIAL AMYLOIDOSIS WITH POLYNEUROPATHY

During the last few years several patients with primary familial amyloidosis with polyneuropathy have been observed in the Umeå hospital region comprising the Northern half of Sweden So far in 13 cases the diagnosis has been established by histological examination In 10 additional patients belonging in the same two families signs and symptoms suggestive of this disease have been observed.

The present case was a 55 year old woman showing progressive sensim tor peripheral neuropathy beginning in the lower extremities She also had malabsorption and vitreous opacities The diagnosis was established ante mortem by examination of biopsy specimens She died after about 6 years of illness at the age of 61

Post mortem examination revealed amyloidosis of peri-collagen distribution with deposits in the vessels of most tissues and organs In peripheral nerves there were important deposits of amyloid but nothing could be detected in the central nervous system Gross deposits were found in the *muscularis mucosae* and in the *tunica muscularis* of the gastrointestinal tract The opacities of the vitreous were shown histochemically to be amyloid Only slight deposits were found in the spleen and the liver The kidneys were moderately involved In the myocardium the amyloid was interstitially deposited Abundant deposits were found in the skin especially in relation to the sweat glands and in the arrector pili muscles.

The diagnosis of this type of amyloidosis is best established by biopsy of skin etal mucosa and peripheral nerves.

Mikulow ki I & B rge Th ALVEOLAR RHABDOMYOSARCOMA

Three ca es f alveolar rhabdomy sarcoma were rep rted In one of the cases a w man 34 years ld the tum ur was located in the breast As far as we kn w this [illegible] n t been lescribed bef re In ther ca e was a 3 year-old b y with generaliz d gr wth f mixed embry nal and alveolar rhabdomyosarcoma The third ase wa a b y 2 years ol l who was well and with ut signs f tum ur 4 years after di gn s A detaile l de cription f the path l gy was given and the differential liagn s wa discu ed The finding were orrelated with literature cases

Publ in A ta path microbi l scand a 232 290 1969)

Ol l ng I GASTRIC ULCERA OF NEWBORN INFANTS

U l e a f the st mach f f ur new b rn infants (three boys and one girl) dying n natally are described The time of survival varied between 7 and 50 hrs and birth weight aried between 1f 0 and 3470 gm In two cases there was Rh in mpatibility with evere immunization of the children and exchange transfusions wer perf rmed soon after delivery In another case the mother got appendicitis n l wa perated n one day bef re delivery and the infant showed signs of severe

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(Publ in Acta path microbiol scand 75 282–290 1969)

Olding L GASTRIC ULCERA OF NEWBORN INFANTS

Ulcera of the stomach of four newborn infants (three boys and one girl) dying neonatally are described The time of survival varied between 7 and 50 hrs and birth weights varied between 1670 and 3470 gm In two cases there was Rh incompatibility with severe immunization of the children and exchange transfusions were performed soon after delivery In another case the mother got appendicitis and was operated on one day before delivery and the infant showed signs of severe

immaturity The fourth infant had a severe cardiac malformation In all cases multiple gastric ulcera were found at autopsy In one case (of Rh incompatibility) there was also perforation peritonitis and septicaemia due to Klebsiella bacteria The other three cases showed no perforation and the autopsy cultures were negative There was no or very little inflammation around the ulcera except in the case of perforation Two types of ulcera were revealed In two cases extensive haemorrhages in the gastric wall and haemorrhagic necroses around the ulcers were found In the other two cases there was a curious homogenization of the connective tissue at the bottom of the ulceration and silver impregnation revealed extensive necroses of the reticular fibers probably due to peptic action of the gastric juice No notable haemorrhages were seen in these two cases.

Grant C A Eriksson B Holm A I Ivemark B & Wallgren C THE ANATOMICAL FINDINGS IN SO CALLED HYPOPLASTIC LEFT HEART SYNDROME

Schnürer L B Fritjofsson A Lindgren A Magnusson P H & Pettersson S FINE NEEDLE VERSUS COARSE NEEDLE IN PUNCTION DIAGNOSIS OF PROSTATIC CARCINOMA

In 163 cases of suspected prostatic carcinoma fine needle punction transrectally according to *Franzén* and coarse needle punction through perineum according to Veenema was performed on the same occasion and the histological and cytological findings were compared

Histologically 85 cancers (52 per cent) were found and the frequency increased with age from 35 per cent (50-59 years) to 78 per cent (80 years and more) A complete agreement between histology and cytology was found in 87 per cent of the subjects if suspicious cases were included among the cancer cases the figure is 91 per cent Among 83 histologically proven carcinomas 76 were diagnosed cytologically and another 2 were suspected One case was cytologically negative and three smears were too scanty Later on three of the cytologically positive but histologically suspicious or negative cases were shown to have cancer

Considering the good agreement between results obtained by the two methods and the fact that fine needle technique is less inconvenient to the patient and gives fewer complications than the coarse needle method the authors recommend the fine needle aspiration technique in cases of clinically suspected carcinoma

Cranberg Ingrid PROSTATIC ASPIRATION BIOPSIES FROM PATIENTS WITH AND WITHOUT SYMPTOMS OR ABNORMAL PALPATORY FINDINGS FROM THE URINARY TRACT—A COMPARATIVE INVESTIGATION

Akerman M Brunk U Hallböök T & Stormby N G FINE NEEDLE BIOPSIES OF PANCREAS DURING OPERATION

Dahlgren S E & Ovenfors C O ASPIRATION BIOPSIES OF INTRATHORACIC NEUROGENIC TUMOURS

Schnürer L B & med kand Wittbolt S AN ADP SYSTEM FOR GYNAECOLOGICAL HEALTH CONTROL

than those in the lower part of the gradient as well as those in the pellet These enzymes included NADH and NADPH cytochrome c reductase NADH ferricyanide reductase and IDPase The highest specific activity of G 6 Pase was found in the fractions exhibiting a sedimentation velocity which was somewhat higher than that of the top fraction In contrast both the AMPase and Mg^{++} ATPase activities gradually increased from the upper to the lower part of the gradient and reached their maximum in the pellet These results strongly indicate the complex nature of the smooth microsomes and possible specialization of the functions within these membranes of the cytoplasm

Jakobsson S V COMPOSITION AND FUNCTION OF KIDNEY CORTEX MICROSOMES

The epithelial cells in kidney cortex have well developed plasma membranes as well as endoplasmic reticulum and it is therefore to be expected that the composition of the total microsomal fraction from the cortex should be complex The composition of the cortical microsomes was investigated by subjecting them to a subfractionating procedure After homogenization of the cortex in diluted tris buffer the membranous elements of the mitochondrial supernate were *concen*trated by centrifugation The suspension layered on a Ficoll gradient range 0–9 per cent in 10 per cent sucrose was centrifuged in a SW 25 rotor at 46 000 g for 60 min

10–15 per cent of the total microsomal protein was recovered in the pellet and 50–55 per cent remained in the upper *part of the gradient* The fractions exhibited a similar PLP/protein ratio but the cholesterol/PLP ratio was higher in the bottom part of the gradient For enzymic analysis the gradient was *divided into pellet* and four subfraction The membranes of the two fractions con*stituting the top* of the gradient display on a protein basis high specific activities for the enzymes catalyzing the oxidation of *NADH and NADPH—the* former including the activities of flavoprotein NT reducta e and cyto*chrome c reductase* as well as the amount of cyt hrome b and the latter measured as the *activities* of flavoprotein NT reductase enzymic lipid peroxidation and the am unt of cytochrome P 450 These fractions also di play an enrichment in IDPa e *activity* The pellet and the fraction bove it have sho n a concentration (up to 80–90 per cent) of AMPase, Mg Na K ATPa e and p NPPase G6Pase activity in general, corresponds to the di tribution of electr n transport enzymes but a *minor* part of th hydrolytic activity is al o attributable to the membranes in the other *fractions*

It appears that the plasma membrane *fragments* of the microsomal fraction of kidney cortex exhibit a high concentration *in the* pell t on a Ficoll gradient In contrast the inner cytoplasmic membranes have a much broader *sedimentation* pattern situated with the *highest specific* activity close to the top of the gradient

Brunk U & Ericsson J L E FINE STRUCTURAL AGE CHANGES IN PONTINE NERVE CELLS

Nerve cells are suitable for studi s of cellular age changes in mammals The age of the cells can be fairly accurately *determined* since they become differentiated early during embr onic development and apparently do not divide after birth

The present stud was un lertaken with *the aim* of studying the cellular alterations accompanying the accumulati n of lipofuscin *in* ageing nerve ells with particular attention to the role of lysosomes and lysosomal enzymes *Sprague*

Dawley rats 3 weeks to 24 months old were anaesthetized with Nembutal and the brains were fixed by perfusion with a prewarmed (37 C) 1 5 per cent cacodylate buffered glutaraldehyde solution Acid phosphatase was demonstrated by incubation of ~ 50 μ thick sections of glutaraldehyd fixed tissues in a modified Gomori medium

Electron microscopy of postosmicated thin sections revealed the occurrence of numerous lipofuscin like acid phosphatase positive cytoplasmic bodies in pontine nerve cells of old (19 to 24 months) rat In young (3 to 8 weeks) rats such bodies were few or absent cytoplasmic organelles carrying acid phosphatase (and presumed to represent lysosomes) were comparatively few and small and had a finely granular matrix containing occasional membranous profiles and/or homogeneous globular structures Cellular autophagocytosis was noted at all ages Apparent transitional stages between cytosegresomes (autophagic vacuoles) and lipofuscin bodies were often observed Acid phosphatase was demonstrated in fenestrated Golgi associated cisternae and also in coated" vesicles (primary lysosomes) which seemed to be budding out from these cisternae

The observations indicate (1) that lipofuscinlike bodies are lysosomes (residual bodies) (2) that these bodies are—at least in part—created through cellular autophagy (3) that there is a continuous new production of lysosomal enzymes throughout the life span of the cells and (4) that newly formed enzymes are enclosed in coated vesicles budding off from Golgi associated cisternae (and presumably represent primary lysosomes) The accumulation of lipofuscin granules may result from an inability of nerve cells to rid themselves of waste products accumulating in residual bodies

Sourander P & *Olsson Y* HISTOLOGICAL CHANGES OF THE SYMPATHETIC NERVOUS SYSTEM IN DIABETES MELLITUS

Ponlén J & *Hugosson R* LONG TIME CULTURING OF NORMAL AND NEOPLASTIC HUMAN CELLS

Westermark B GROWTH CONTROL OF NORMAL AND MALIGNANT GLIA *IN VITRO*

Normal human glia cells in tissue culture are extremely sensitive to proximity depending cell cycle inhibition which means that the cell division stops when the cells have formed a confluent monolayer

The degree of cell cycle inhibition among normal and malignant glia cells has been studied in co cultivation experiments

In experiment one normal glia cells were seeded on top of a preformed stationary monolayer of glia cells As a control glia cells were seeded alone Cell count revealed that the top cells were completely inhibited by the bottom cells while the glia cells seeded alone proliferated logarithmically

In experiment two cells from the established glioma line 138 MG were seeded on top of a preformed monolayer of glia cells A control consisted of glioma cells alone The glioma cells proliferated logarithmically with the same rate when seeded alone or on glia cells

These experiments show that a confluent glia monolayer inhibits normal but not malignant glia cells Whether this reflects a qualitative or quantitative defect in the growth control of the neoplastic cells is not known

Lagerlöf P & Sundelin I ELECTRONMICROSCOPICAL CHARACTERIZATION OF HAEMOLYSIN PRODUCING CELLS IN MYELOID LEUKAEMIA IN FOWL

The viru induced myeloid leukaemia in fowl can be superimposed by anaemia f haemolytic type Some of the animals sh w transient positive Coombs test In order to elucidate whether these findings reflect an actual disturbance of the immunological behaviour of the chicks we have tested the antibody producing capacity of various cells of the leukaemia chic by use of the Jerne haemolytic plaque technique The cells from pl en and peripheral bl od of some of the leukaemic chi ks were shown to be as a tive antibody producers as those from non leukaemic chicks after antigenic stimulation with sheep red blood cells Electron microscopical analysis showed that the plaque forming cells were plasma cells in all the non leukaemic case Similar cells were found in some of the plaque from leukaemic chicks In some cases the cells in the centre of the plaque were indi tinguishable ultrastructurally from myeloid leukaemia cells however The implications of these findings were dis ussed

Nilsson K LYMPHOBLASTOID TRANSFORMATION IN LONG TERM CULTURE OF HUMAN TISSUE

The term lymphoblastoid transformation (l tr) (*Deny sh M Intel* 1963) describes the establishment *in vitro* of permanent lymph id cell lines from human lymph id tis ue Until 1967 l tr was considered to occur onl *in cultures of malig* nant lymphoma of leukaemic tis ue The incidence of l tr *in different* tissue culture systems has been reported to be very low Previously (Int J Cancer 3 183–190 1968) we reported the best results so far (50 per cent) obtained *in cultures of* normal and malignant lymph nodes We use a modified Trowell Jensen organ culture and can now report l tr in 17 consecutive specimens from normal *human* lymphoid tissues Morphologically the established cell lines consist of blastoi l cells in the lymphoid series The cells depend on fibroblasts for their *survival* and preserve a highly differentiated function refle ted by a mon clonal *production* of IgG or IgA (1 case) or IgM (1 case) The light chain is always kappa

Since infinite growth *in vitro* is considered a criterion *of malignancy* for somatic human tissue it is remarkable that permanent cell lines from human lymphoid tissue can be established in perhap 100 per cent *To explain* this finding three hypotheses are presented

A Normal lymphoid ti sue c ntains some cells capable of infinite proliferation

B Normal lymphoid tissue contains neoplastic cell clones controlled *in vivo* by unknown *mechanisms*

C Infinite growth of n rmal lymphoid cells is *ind iced in vitro* by unknown agent (virus? antigen?)

Olding I MATERNO FOETAL TRANSFER OF LYMPHOCYTES IN HUMAN SUBJECTS

Tran pla ental transfer f lymphocytes from mother to foetus was tudied by searching for cells with a female karyotype in the *cord blood* of newborn boys The leucoc te culture technique of Moorhead et al (1960) w s employed. Only meta phases with 46 chr mos mes were included in the *study* Up to now eight newborn b s have been examined In two of them lymphocytes with a female karyotype ere dis overed In one case one cell out of 135 was *female* and in the other ase 2

cells out of 145 In the other six cases altogether 4o3 metaphases were analysed no female karyotype was found In these six cases however the total number of examined cells per individual varied between 29 and 177 because of varying quality of the chromosome preparations and it is possible that the frequency of trans placental cell transfer might have been larger than the one indicated by the result ing figures (2/8) if a larger number of cells could have been analysed The theo retical consequences of maternofoetal chimerism might be the production of immunoglobulins by maternal cells in the foetus or the foetal ability of delayed hypersensitivity reactions acquired from the mother

Hagmar B & Boeryd B STUDIES ON THE EFFECT OF HEPARIN ANTIPRO THROMBIN AND EPSILON AMINOCAPROIC ACID ON THE FORMATION OF SPONTANEOUS METASTASES

Anticoagulants and antifibrinolytics have been described to decrease and increase respectively the formation of metastases in allogeneic systems The effects of heparin antiprothrombin (phenprocoumon Marcoumar) and epsilon aminocaproic acid (EACA) were now tested on the formation of spontaneous metastases from resectable syngeneic tumours transplanted to the tails (20 methylcholantrene in duced rhabdomyosarcoma MCG1 SS in CBA mice) The periods of treatment six days were varied in order to affect different phases of the metastatic process The metastasis dissemination started between day 7 and 13 after transplantation and treatment was first instituted between these day followed by resection of the tumours Heparin increased the average and total volumes of metastases to the lungs phenprocoumon decreased the number and total volume while EACA de creased the average and total volumes Resection on day 13 followed by treatment during 6 days revealed no differences between treated groups and controls In stitution of treatment on day 1 followed by resection on days 5 7 and 9 gave a higher incidence of pulmonary metastases only in heparin treated animals resected on day 5 Heparin increased the spontaneous metastasis formation while phen procoumon decreased it Heparin seems to promote an earlier release of tumour cells from the tumours EACA reduced the formation of spontaneous metastases

Norrby K Boeryd B Knutson F & Lundin I M EXPERIMENTAL STUDIES ON CIRCULATING TUMOUR CELLS

Factors influencing the pattern of secondary metastatic tumour growth are clinically as well as experimentally poorly understood In two murine syngeneic tumour host systems MCG 1 SS (solid) and AA (ascites) CBA and Melanoma B 16/C57Bl the number of monodispersed tumour cell required for progressive tumour growth after intravenous intraperitoneal and subcutaneous transplantation was determined In both systems about 10^3 more cells were required for successful intravenous transplantation compared to subcutaneous and intraperitoneal trans plantations

Intravenously transplanted ^3H thymidine labelled MCG 1 SS and AA tumour cells (label index about 85) were studied by radiochemical and autoradiographic techni ques Most of the injected cells were primarily trapped in the lungs However the DNA bound isotope activity in the lungs decreased quickly After 6 hours about 15 per cent of the injected activity remained in the lungs after 24–48 hours only a few per cent The activity decreased at the same rate in other tissues including the liver

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The fact that considerably more cells are required for successful transplantation intravenously than subcutaneously and intraperitoneally is probably due to an extensive and rather quick disintegration of intravenously transplanted cells. This disintegration of highly viable monodispersed syngeneic tumour cells in the circulation is thus far unexplained. These matters are being additionally studied.

Lindberg L. G. VIRUS PARTICLES IN ROUS SARCOMA OF HAMSTERS

Ericsson J. L. E. GLUCAGON INDUCED CELLULAR AUTOPHAGY IN HEPATOCYTES

Intraperitoneal administration of crystalline glucagon to rats (50 μg/100 g of body weight) promptly induces greatly enhanced cellular autophagy in hepatocytes. Previous studies of such cells containing prelabelled heterophagic secondary lysosomes (thorotrast or iron labelled) indicated that forming and early cytosegresomes (autophagic vacuoles) lacked lysosomal enzymes and acquired these enzymes by merger with pre-existing lysosomes, usually of secondary type.

The present investigation was conducted in order to elicit the origin of the membrane(s) constituting the wall of early cytosegresomes. The livers of glucagon treated rats were fixed by perfusion with 1.5 per cent buffered glutaraldehyde and 50 μ thick frozen sections were incubated in appropriate substrates for the fine structural demonstration of typical plasma membrane, endoplasmic reticulum and Golgi enzymes. These included adenosine triphosphatase (ATPase) for plasma membrane, glucose-6-phosphatase (G-6-Pase) and inosinediphosphatase (IDPase) for endoplasmic reticulum and thiamine pyrophosphatase (TPPase) for Golgi membranes. High resolution electron micrographs of thin sections of Vestopal embedded tissues were taken with special references to the ultrastructure of those membranes which appeared to be involved in the segregation of cytoplasmic organelles in glucagon treated animals.

The findings indicated that the membranes surrounding forming cytosegresomes were derived from the endoplasmic reticulum since they showed activity of G-6-Pase and IDPase and were triple-layered with a thickness of ~ 60 Å. ATPase activity was not demonstrated in these membranes. TPPase was present both in Golgi and endoplasmic reticulum membranes of control and experimental animals and in membranes participating in the sequestration of cytoplasmic organelles in animals given glucagon.

Medlund, Hansson H. A. & Sourander P. ELECTRON MICROSCOPICAL INVESTIGATION OF LYSOSOMES IN TOXOPLASMA GONDII

Ljungqvist A. THE INTRARENAL VESSEL ARCHITECTURE IN EXPERIMENTAL HYPERTENSION

Falkner S., Hellerström ... & Hellman B. SULPHHYDRYL GROUPS AND PANCREATIC ISLET TISSUE

In order to test the validity of the sulphhydryl (SH) theory for the pathogenesis of alloxan diabetes the following experiments were made.

Assays of the glutathione (GSH) content in microdissected mammalian pan

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creatic islets with 80 90 per cent β cells by a micromodification of the orthoph thalaldehyde procedure showed that the CSH content of the islet parenchyma was higher (20 30 mg/100 g) than that of the acinar tissue (10–15 mg/kg) in all species studied (man rats AO mice)

Feeding non diabetic Chinese hamsters a diet deficient in cystein methionine did not increase the low frequency of spontaneous diabetes mellitus that occurs in this species but evoked a pathological glucose tolerance in 90 per cent of the animals Moreover in 2 hamsters (of 38) degranulation and necrosis were observed in some islets selectively affecting the β cells

Dithiol inhibitors as CoCl CdCl and $NaAsO_2$ were found to be able to evoke hyperglycaemia in the daddy sculpin and selective necroses in the dark central region of the principal islets where the β cells occur However no CSH lowering effect like that obtained after alloxan was observed

Excessive methionine administration (0 5 g/kg i p for 2 week) supposed to produce an SH compound imbalance was found to evoke atrophy of the exocrine pancreatic parenchyma in the Chinese hamster In 2 animals (of 4?) selective β cell necrosis was observed in the islets

Though the CSH experiments did not conform to the SH theory the other observations indicate that SH compounds are of greater importance for the β cells than for the other islet parenchymal cells

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RETICULOENDOTHELIAL ACTIVITY RELATED TO AGE AND SEX IN MICE

By

F Hartveit and K Andersen

Received 1 xi 68

In the course of experiments preliminary to work on the measurement of clearance time following the injection of carbon particles in mice (1) it became evident that female mice were able to clear such particles quicker than males of the same age This unpublished observation was followed up and the results are presented here

MATERIAL AND METHODS

Mice of the closed colony kept at this Institute were used The numbers used their age and body weight are shown in Fig 1

The carbon clearance time was determined after the intravenous injection of a suspension of Pelikan ink (C11/1431a) in physiological saline containing 1 per cent gelatine Blood samples were taken at timed intervals after injection and examined microscopically for carbon as described previously (1) the dose used being equivalent to 8 mg/100 g body weight

After determination of the clearance time the mice were killed and the liver and spleen removed and weighed

RESULTS

Clearance time The results are given in Table 1 which shows that at the age of 1 month the clearance time was similar in both sexes The clearance time then increased in both sexes with increasing age but this increase was much more marked in the males than in the females the sex difference being statistically significant at 3 and 6 months $(0.01 > P > 0.001$ and $0.001 > P)$

Organ weights Fig 2 shows that the liver weight increased with age The spleen weight was greater at 6 than at 1 month but showed great variation at 3 months (Fig 3) In both cases the male values tended to be greater than the female values but significant differences were not found The relative organ weight was similar in both sexes and tended to decrease with age (Table 2)

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* Research Fellow Norwegian Cancer Society

TABLE 1

Mean Carbon Clearance Time (± SD) Related to Age and Sex in Mice

Age (months)	Clearance time (mins)	
	Male	Female
1	13 2 ± 7	19 1 ± 6
3	39 3 ± 6	28 6 ± 3
4	57 4 ± 8	33 2 ± 6

For number of mice see Fig 1

TABLE 2

Relative Organ Weight Related to Age in Mice

Age (months)	Organ weight (mg/g body weight)			
	Liver		Spleen	
	male	female	male	female
1	[illegible]	61	4	5
3	[illegible]	[illegible]	3	2
6	48	50	3	3

For number of mice see Fig 1

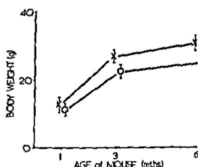


Fig 1

Body weight related to age and sex in the mice used in the present experiments Male values X female 0 ± SD

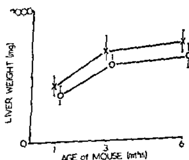


Fig 2

Liver weight related to age and sex in the mice used in the present experiments (For number of mice see Fig 1) Male values X female 0 ± SD

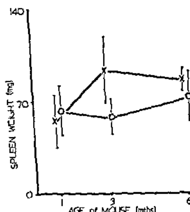


Fig 3
Spleen weight related to age and sex in the mice used in the present experiments (For number of mice see Fig 1) Male values X female O ± S D

DISCUSSION

Reticuloendothelial activity is commonly measured by determination of the time required for the removal of inert particles from the blood stream Such particles are taken up by phagocytic cells in all parts of the body—but the majority are cleared from the circulation by the Kupffer cells of the liver and their counterparts in the spleen As the size of these two organs and their relationship to total body weight changes with age it is reasonable to expect this to influence the clearance time when dosage is given according to total body weight as in the present experiment The increase in clearance time with increasing age in both sexes can probably be in part explained in this way The sex difference in clearance time can however not be accounted for on this bases as the relative organ weights were remarkably similar in both sexes The difference appeared only after the mice had reached maturity This coupled with the finding that oestrogens may cause an increase in phagocytic activity in male mice (2) makes it likely that the differences observed in the present experiment are the result of hormonal action

SUMMARY

The clearance time for carbon particles in mice increases with age and is less in adult female than male mice The latter is probably due to the stimulating effect of oestrogens on phagocytosis

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SALICYLATE-INDUCED FOETAL HAEMORRHAGE IN TWO MOUSE STRAINS

By

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Received 15 xi 68

In teratological experiments with salicylates a high rate of foetal resorption has been found (*Warkany & Takacs* 1959 *Goldman & Yakovac* 1963 *Klein Obbink & Dalderup* 1964 *Larsson & Bostrom* 1965) An interesting observation in earlier experiments on mice was the increasing incidence of foetal mortality the later in pregnancy salicylate was administered to the mother (*Larsson & Eriksson 1966*) The possible mechanisms underlying the salicylate induced prenatal mortality were discussed Haemorrhage has been suggested to cause foetal death when acetylsalicylic acid has been administered to pregnant rats (*Brown & West* 1964)

In further investigations in our laboratory with special reference to drug induced late foetal death a high incidence of superficial haemorrhage was found in the foetus when salicylate was given to the mother on the 16th or 17th gestation day Moreover a special form of subcapsular haemorrhage in the liver attracted our attention when the abdomen of the foetus was opened for inspection of the viscera

The present study was undertaken for further investigation of these observations and of their possible relation to foetal mortality

It also seemed of interest to compare in this respect two mouse strains with a known difference in their susceptibility to salicylate (*Larsson & Bostrom* 1965)

MATERIALS AND METHODS

A totally of 64 pregnant primiparous mice of the A/Jax strain and 52 of the CBA strain were used They were mated overnight and on the following morning vaginal plug could easily be observed in most cases (*Larsson* 1962) This day was denoted as zero day of pregnancy

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I take this opportunity of expressing my sincere gratitude to Dr *K Sune Larsson* for valuable advice during preparation of the manuscript I also wish to thank Mr *Lennart Nilsson* for photographical assistance and Mrs *Tollie Palm* and Miss *Eva Löfgren* for skilful secretarial and technical assistance

The expenses of the investigation were defrayed by grants from Reservationsanslaget for framjande av medicinsk forskning from the *Swedish Medical Research Council* (14X–993–02 and 14P–993–02) and from grants to Drs *K S Larsson* and *H Boström* from the *Association for the Aid of Crippled Children* N Y *USA*

TABLE 1

Effect of Sodium Salicylate on Foetal Mortality, Superficial and Liver Haemorrhages in the Foetuses, the Drug being Administered to the Mother in a Single Dose of 10 mg/20 g on Gestation Day 16, 17 or 18

Strain	Gestation day injected	Time of sacrificing after inj hrs	No of litters	No of foetuses	Foetal mortality		Superficial haemorrhage in living foetuses		Liver haemorrhage in living foetuses	
					no	%	no	%	no	%
A/Jax	16	8	7	48	22	46	11	42	5	19
CBA	16	8	5	39	1	3	18	49	4	11
A/Jax	16	24	7	46	20	43	15	56	7	26
CBA	16	24	8	46	3	7	9	21	0	0
A/Jax	17	2	6	36	0	0	1	3	1	3
A/Jax	17	4	6	40	0	0	14	35	1	3
CBA	17	4	6	36	0	0	12	33	1	3
A/Jax	17	8	7	47	9	19	8	21	6	16
CBA	17	8	7	38	2	5	20	56		6
A/Jax	17	12	7	50	27	54	12	52	7	30
CBA	17	12	7	37	9	24	10	36	0	0
A/Jax	17	24	10b	49	19	39	6	20	6	20
CBA	17	24	9	31	4	13	2	7	0	0
A/Jax	18	8		51	0	0	0	0	1	2
CBA	18	8	5	33	0	0	0	0	0	0
A/Jax	18	24	7d	—	—	—	—	—	—	—
CBA	18	24	5	—	—	—	—	—	—	

a 1 delivered before dissection. b 2 delivered before dissection. c 3 delivered before dissection. d All 7 delivered before dissection. All 5 delivered before dissection. Newborns were not examined and are therefore not included.

Sodium salicylate (10 mg/10 g body weight in 0.1 ml of distilled water) was given i.m. in a single dose at 10 a.m. on gestation day 16, 17 or 18. The mice injected on the 16th and 18th days were sacrificed 8 or 24 hours after treatment and the foetuses were removed. The mice injected on the 17th day were sacrificed after 2, 4, 8, 12 or 24 hours (Table 1). If the female delivered before sacrificing this was recorded and the newborns were not examined.

The foetuses were divided into dead and alive. Early foetal resorptions which had evidently occurred before the mother had received the injection were not recorded.

Superficial haemorrhage and its exact site were noted. The abdomen was opened and the liver was inspected in situ for haemorrhage. The involvement of different lobes by the haemorrhage was recorded. Foetuses found dead in the groups sacrificed 24 hours after injection were, however, too severely macerated to be properly examined. The whole foetus was immersed in Bouin's fluid and on the following day the liver was removed and once more examined.—The mother's liver was also inspected before and after fixation.

The livers of at least two foetuses from most litters were taken for paraffin embedding, sectioning at 7 μ and staining with haematoxylin and eosin.

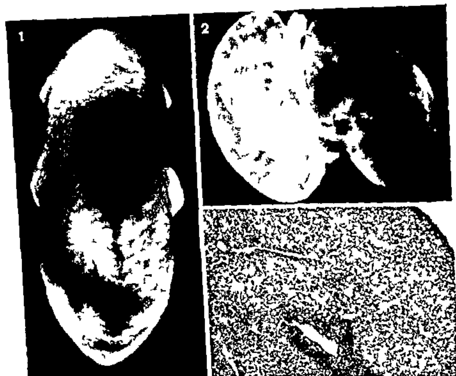
RESULTS

Foetal Death

The incidence of foetal mortality is expressed as the ratio of foetuses dead late in pregnancy to the total number of foetuses living and later dead (Table 1). In the A/Jax strain the maximum mortality of about 50 per cent was reached already 8 hours after injections given on the 16th day. If given on the 17th day, foetal death of 19 per cent was found first 8 hours after injection, increasing to a maximum of 54 per cent after 12 hours. In the CBA strain foetal death was seldom noted if injection was given on the 16th day, i.e. at most 7 per cent. If injections were given on the 17th day the incidence of foetal death was lower than that in the A/Jax strain, the maximum of 24 per cent being reached also 12 hours after injection. After injection on the 18th day no dead foetuses were observed in either strain. A tendency to premature delivery within 24 hours of injection was generally found after administration on days 17 and 18.

Superficial Haemorrhage

The incidence of superficial haemorrhage is expressed as the ratio of living foetuses with superficial haemorrhage to the total number of live foetuses (Table 1). The haemorrhage observed was situated along the spine (Fig. 1). In the A/Jax strain superficial haemorrhage was found if the drug was administered on the 16th day, increasing from 42 per cent after 8 hours to a maximum of 56 per cent after 24 hours. If administered on the 17th day the incidence was 35 per cent after 4 hours, increasing to a maximum of 56 per cent after 12 hours. In the CBA strain superficial haemorrhage was noted at almost the same rate as in the A/Jax strain. If injections were given on the 16th day, however, the maximum of 49 per cent was already reached at 8 hours with a decrease to 21 per cent at 24 hours. If given on the 17th day as well, the maximum incidence of 56 per cent was reached after 8 hours



Figs 1–3

Fig 1 Superficial haemorrhage along the spine in a 17 day old A/Jax mouse foetus The mother was given sodium salicylate i m (10 mg/20 g) 8 hrs prior to sacrifice

Fig 2 Varying degrees of liver haemorrhage in a 17 day old A/Jax mouse foetus The mother was given sodium salicylate i m (10 mg/20 g) 8 hrs prior to sacrifice

Fig 3 HTX and eosin stained 7 μ section from the liver of a 17 day old A/Jax mouse foetus The mother was given sodium salicylate i m (10 mg/20 g) 12 hrs prior to sacrifice Normal liver cells are seen around the vessels Haemorrhages are visible subcapsularly × 60

All dead foetuses that could be examined had superficial haemorrhage

After injection on the 18th day no superficial haemorrhage was observed in either strain

Liver Haemorrhage

The incidence of liver haemorrhage is expressed as the ratio of living foetuses with such haemorrhage to the total number of live foetuses (Table 1)

The liver haemorrhage was seen subcapsularly and macroscopically did not extend into the parenchyma (Fig 2) Histological examination revealed necrosis and haemorrhage extending into the parenchyma

from beneath the capsule (Fig 3) In a few cases focal necrosis was also present in microscopically unaffected foetuses

In the A/Jax strain liver haemorrhage was *found in approximately half of the living foetuses with superficial haemorrhage* on the 16th *day On the 17th day* only one foetus (3 per cent) had liver haemorrhage after 4 hours the incidence increasing to 30 per cent after 12 hours In the CBA strain on the other hand liver *haemorrhage was only occasionally found* on both days the maximum incidence being *11* per cent 8 hours after injection on the 16th day All dead foetuses that could be examined—except one 16 day old CBA foetus and two 17 day old CBA foetuses taken 8 hours after injection—had liver haemorrhage generally involving all lobes

After injection on the 18th day liver haemorrhage and histologically demonstrable necrosis were present in only one A/Jax foetus

DISCUSSION

The main question in this investigation is obviously the possible relation between the haemorrhages and the high foetal mortality Such a relation is indicated by the observation that a high percentage of foetal haemorrhage precedes the increased incidence of foetal death Moreover the fact that all dead foetuses which could be examined showed superficial haemorrhage and all but 3 CBA foetuses liver haemorrhage supports the view that haemorrhages are involved in the foetal death There were however almost always living foetuses with haemorrhage Whether or not these would have died later or whether their damage was less severe and might have been overcome can only be speculated

In view of the fact that the maximum mortality on the 17th day was as high as 54 per cent in the A/Jax strain– although death occurred somewhat later than on the 16th day—it is interesting to note that foetuses exposed to the drug on the 18th day were only rarely affected by the salicylate This difference in the susceptibility to salicylate may perhaps reflect the maturation of the foetus own detoxification enzyme system Drugs administered to the mother have been shown to affect the foetal liver enzyme activity (*Gordon et al* 1961 *Ordy et al* 1966) or to cause necrosis of the liver and delay its maturation (*Knoll & Vilmar* 1963) *This could presumably* also occur in the salicylate affected foetal liver with haemorrhage and necrosis

It is also interesting to observe that even if the CBA foetuses had superficial haemorrhage to almost the same extent as the A/Jax foetuses most of them seemed to survive The mechanism of this strain difference is not known

Another interesting feature is the presence of haemorrhage in the foetus but never in the mother The haemorrhage *seems to resemble the* petechial haemorrhages which have been observed in salicylate poison

ing, i.e. it is present subcutaneously below the serous membranes and in a variety of organs (*Smith & Smith* 1966). Drugs such as quinine, thiazide and coumarin have been reported to cause haemorrhage in the foetus or newborn when administered to the mother (*Posner* 1937, *Rodriguez* 1964, *Mer. & Breitner* 1936). Salicylates and phenobarbital in excess have been stated to cause neonatal haemorrhage (*Apgar* 1964).

SUMMARY

Sodium salicylate in a single dose of 10 mg/20 g was given to pregnant mice of the A/Jax and CBA strains on gestation days 16, 17 or 18, and they were sacrificed at various intervals after injection.

Foetal death occurred after injection on the 16th and 17th day. In the A/Jax strain the maximum mortality of about 50 per cent was reached earlier if injections were given on the 16th day (after 8 hours) than if they were given on the 17th day (after 12 hours). The incidence of foetal death was markedly less in the CBA foetuses.

Superficial haemorrhage was observed in all dead foetuses and in about one half of the living ones after injection on the 16th and 17th day. This applied to both strains.

In the A/Jax strain liver haemorrhage with histologically demonstrable necrosis was present in all dead foetuses and in about one half of the living ones with superficial haemorrhage. This was rare in the CBA strain.

After injection on the 18th day foetuses seem only rarely to be affected and only those in the A/Jax strain.

The possible relation between foetal mortality and induced haemorrhage is discussed.

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MULTIPLE PRIMARY MALIGNANT TUMOURS

An Autopsy Study of a Circumscribed Population

By

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the true number of multiple cases can be estimated only by following the patients up as carefully as possible to the end of their lives and by examining all of them post mortem (Malmio 1959)

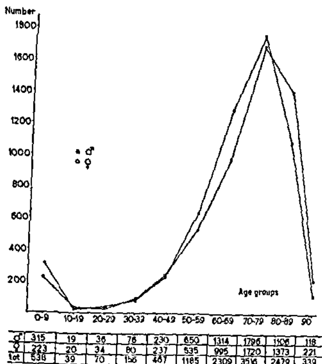
This is of course the ideal method but unfortunately very difficult to carry out Another possible way is to make use of an autopsy material and by means of the clinical records and the files of the *department* of pathology study malignancies earlier diagnosed in these subjects This is the method applied in this work and since we have been able to perform autopsy on about 60 per cent of those in a well defined population who died during the nine years 1958–1966 we think it worth while to describe the frequency of multiple cancer in these subjects

There are however many problems associated with a study of this type A patient with a slowly growing tumour like most of the prostatic carcinomas runs a greater risk of developing another cancer than for instance those with a malignant melanoma The results ultimately depend on diagnostic and therapeutic possibilities which may explain the different frequencies reported in clinical and autopsy series

In a later publication we will describe in detail some of the more frequent tumours where it is possible to evaluate the morbidity risk at different ages the frequencies of correct clinical diagnosis and the changes of survival It should then be possible to compare the expected frequencies of multiple tumours with those found

MATERIAL AND METHODS

Malmö is a town situated in the *south of Sweden* In 1966 it had about 250 000 inhabitants The town offers unusually good possibilities for medico demographic studies because it has a well defined population and as far as the medical service is concerned is a seperate region The town has only one general hospital (Malmo General Hospital) one infirmary *for chronic diseases* one hospital for mental diseases and only one department of pathology serving the entire area Out of the 18 675 *who died during* the e years 11 098 where autopsied, *i e* 59 4 per cent The age and sex distribution of the material is given in Fig 1 The autopsies were performed according to uniform principles which are described *in* some detail earlier (*Berge* 1967)



	0-9	10-19	20-29	30-39	40-49	50-59	60-69	70-79	80-89	90
♂	315	19	36	76	230	650	1314	1796	1106	118
♀	223	20	34	80	237	535	995	1720	1373	221
Tot	538	39	70	156	467	1185	2309	3516	2479	339

Fig 1

Age and sex distribution of the 11 098 subjects autopsied 1958–1966

Data concerning previous treatment for cancer were taken from the clinical records which always were accessible before autopsy was performed Further the archives of the Department of Pathology were analyzed in order to see if the patient earlier had been treated for cancer

DEFINITIONS

1 The term multiple tumours was used to designate two or more synchronous or metachronous tumours appearing in different organs The terms synchronous and metachronous were related to the situation at autopsy

2 The tumours were histologically malignant

3 The tumours were not metastases

4 One or more lesions with the same histological appearance (with unequivocal signs of malignancy) in any one organ or paired organs as well as primary generalized tumours (*e g* malignant lymphoma) were said to be one tumour

5 Latent prostatic cancer was included

6 Basal cell cancer was not accepted because of the biological benignity of this tumour and because data on previous treatment often were incomplete or missing

7 The group called *glioma* consists of different types of astrocytoma oligodendroglioma medulloblastoma and ependymoma

8 The term cured in cases treated for cancer is used in the sense *that recurrence* or metastases could not be demonstrated at autopsy

The data were processed with the computer in Lund

Thanks go to fil kand *Arne Sundstrom* Datacentralen Lund for most *valuable* assistance

TABLE 1

Age and Sex Distribution

Age group	Total	Subjects with 1 tumour			Subjects with 2 tumours				Subjects with 3 tumours				Subjects with 4 tumours				Subjects with multiple tumours in per cent of all
		Male	Female	Total	Male	Female	Total	Per cent of all subjects with tumours	Male	Female	Total	Per cent of all subjects with tumours	Male	Female	Total	Per cent of all subjects with tumours	
0–9	16	8	8	16	0	0	0	0	0	0	0	0	0	0	0	0	0
10–19	15	6	9	15	0	0	0	0	0	0	0	0	0	0	0	0	0
20–29	36	24	12	36	0	0	0	0	0	0	0	0	0	0	0	0	0
30–39	80	31	46	77	1	2	3	3.8	0	0	0	0	0	0	0	0	3.8
[illegible]	251	85	159	44?	3	4	7	2.8	0	0	0	0	0	0	0	0	2.8
[illegible]	652?	241?	317	595?	17	30	47	7.2	3	3	6?	0.9	0	1	1	0.2	8.3
[illegible]	1168?	524?	487?	1043?	79	36?	115	9.8	7	1	8	0.7	0	0	0	0	10.5
[illegible]	[illegible]	[illegible]	[illegible]	1127?	117	55?	19?	1 5	12	2	14	0.9	0	1	1	0.07	13.5
[illegible]	[illegible]	[illegible]	118?	883?	90?	45?	175?	11.1	10	6	16	1.5	1	1	2	0.2	14.8
[illegible]	[illegible]	[illegible]	[illegible]	85?	13?	9?	22	20.0	1	1	4?	1.8	1	0	1	0.?	22.7
[illegible]	[illegible]	14?	[illegible]	[illegible]	110?	181?	291?	10.8?	33?	13?	46?	0.9	3	1	5	0.1	11.7

RESULTS

Among the 11 098 autopsied subjects 4 895 (44.1 per cent) had or had had 5 523 primary malignant tumours.

The subjects with single and multiple tumours are given according to age and sex in Table 1.

It is seen from Table 1 that a single malignant tumour occurred in 4 323 subjects and multiple malignant tumours in 572, i.e. 11.7 per cent (521, 10.6 per cent, had 2, 46, 0.9 per cent, had 3 and 5, 0.1 per cent, had 4 tumours.) Tumour was present at autopsy in 4 062 of the subjects with a single tumour and in 561 of those with multiple tumours. Among the 521 subjects with double cancers 377 had both tumours, 133 had 1 and 11 had none at autopsy. Among those with triple cancers 25 had 3, 15 had 2 and 6 subjects had 1, and among those with quadruple cancers 1 had 4 and 4 had 3 tumours at autopsy. (More than 2 cancers had not been cured in any subject.) Multiple tumours synchronous at autopsy were thus found in 422 subjects while the tumours were metachronous at autopsy in 150 subjects.

TABLE 2

Number of Tumours at Different Sites and Types and Frequency of Syn- and/or Metachronous Tumours

Site	Number	Mean age at death	Frequency of multiple tumours				%
			2 tumours	3 tumours	4 tumours	*Total*	
Carcinoma							
Prostate	808	76.5	215	26	2	243	30.1
Colon	482	7[illegible].6	92	12	2	106	22.0
Breast	478	67.4	79	9	1	90	18.8
Lung	475	69.0	83	5	1	89	18.5
Stomach	463	71.5	73	13	1	87	18.8
Kidney	2[illegible]1	70.4	70	13	0	83	33.1
Rectum	199	70.3	40	4	0	44	22.1
Pancreas	196	67.3	38	5	0	43	21.9
Liver	186	[illegible]0.8	28	5	0	33	17.7
Ovary	173	63.9	17	1	1	19	11.0
Biliary system	172	72.2	23	1	1	25	14.5
Urinary bladder + urethra	149	7[illegible].6	21	3	3	27	18.1
Uterine cervix	129	60.8	15	3	0	18	14.0
Small intestine	113	[illegible]3	41	3	0	44	38.9
Uterine body	81	71.6	21	1	2	24	29.6
Oesophagus	78	71	19	0	0	19	24.4
Skin	69	65.8	9	6	1	16	23.2
Thyroid	54	63.8	11	3	1	15	27.8
Unknown	43	66.7	6	0	0	6	14.0
Renal pelvis + ureter	26	69.8	7	0	3	10	38.5
Larynx	21	69.4	5	2	0	7	33.3
Testis	16	43.4	[illegible]	0	0	2	12.5
Vulva	15	71.5	1	1	0	2	13.3
Appendix	14	65.1	2	3	0	5	35.7

TABLE [illegible] (cont.)

Site	Number	Mean age at death	Frequency of multiple tumours: 2 tumours	3 tumours	4 tumours	Total	%
Oral cavity	13	70.[illegible]	4	0	0	4	30.8
Anus	11	67.6	2	0	0	2	18.2
Uterus unspec	10	67.5	2	0	0	2	20.0
Eye	10	69.9	2	1	0	3	30.0
Maxillary sinus	10	75.0	2	0	0	2	20.0
Hypopharynx	9	70.8	1	0	0	1	11.1
Tongue	8	70.9	1	0	0	1	12.5
Lip	8	74.6	3	1	0	4	50.0
Salivary gland	8	73.1	1	0	0	1	12.5
Epipharynx	6	6[illegible].3	0	0	0	0	–
Testis	6	73.5	3	0	1	4	66.7
Adrenal	4	67.5	1	0	0	1	25.0
Vagina	3	80.3	0	0	0	0	–
Tube	3	63.3	0	0	0	0	–
Tonsil	2	66.5	0	0	0	0	–
Sweat gland	1	68.0	1	0	0	1	–
Trachea	1	65.0	1	0	0	1	–
Cardia	1	81.0	0	0	0	0	–
Other tumours							
Leucosis	217	60.5	34	6	0	40	18.4
Glioma	118	59.1	6	2	0	8	6.8
Reticulum cell sarcoma	113	67.3	21	3	0	24	21.2
Myelomatosis	76	72.4	11	1	0	12	15.8
Undiff. mesenchymal tumour	46	61.1	11	0	0	11	23.9
Hodgkin	42	54.2	5	2	0		16.7
Mesothelioma	32	70.3	2	0	0	2	6.3
Leiomyosarcoma	24	73.4	4	2	0	6	25.0
Lymphosarcoma	12	69.8	2	0	0	2	16.7
Thymoma	8	64.4	1	0	0	1	12.5
Liposarcoma	6	78.5	1	0	0	1	16.7
Osteogenic sarcoma	5	37.6	0	0	0	0	–
Meningioma	4	75.5	1	0	0	1	25.0
Ewing sarcoma	4	16.8	0	0	0	0	–
Rhabdomyosarcoma	3	49.7	0	1	0	1	33.3
Chordoma	3	76.0	1	0	0	1	33.3
Brill Symmers	2	63.0	0	0	0	0	–
Mycosis fungoides	2	71.5	1	0	0	1	50.0
Neuroblastoma	1	6[illegible].0	0	1	0	0	–
Total	5593	69.7	1042	138	20	1200	

Treatment for one cancer had been successful in 413 subjects but in 152 of these (36.8 per cent) one or more other tumours were found at autopsy. Six (35.3 per cent) out of the 17 patients cured for 2 tumours were found to have another tumour at autopsy. The frequency of a second tumour increased with time after successful treatment but these problems will be discussed in detail in a later publication.

The series consisted mainly of aged subjects. The average age at death was 68.5 years in those with single cancers, 73.7 years in those

TABLE 3

Number and Locations (Types) of Multiple Tumours

Site	Number	Prostate	Colon	Stomach	Malignant lymphoma	Breast	Kidney	Lung	Uterus	Pancreas	Rectum	Small intestine	Liver	Urinary bladder + urethra	Biliary system	Skin	Ovary	Thyroid	Oesophagus	Renal pelvis —ureter	Larynx	Glioma	Appendix	Penis	Unknown site	All other tumours	Total
Prostate	908	–	33	30	20	0	29	37	–	20	15	13	18	11	6	5	–	3	7	2	3	2	0	3	1	15	273
Colon	482	33	–	13	10	11	4	5	6	4	1	8	3	6	3	0	1	[illegible]	0	3	0	0	1	0	0	3	122
Breast	478	0	13	13	9	[illegible]	8	6	12	5	3	2	8	2	2	4	9	1	0	0	0	0	0	–	0	9	100
Malign lymph	476	20	10	5	12	9	6	4	5	4	4	3	2	1	1	3	[illegible]	1	2	0	0	1	2	0	2	0	99
Lungs	475	37	5	6	4	6	4	2	4	1	3	1	4	2	3	0	0	0	2	0	2	0	0	0	0	4	95
Stomach	463	30	13	–	5	13	9	6	5	2	2	4	[illegible]	1	2	1	1	0	0	1	0	0	1	0	1	3	102
Kidney	251	29	4	9	6	8	–	4	1	4	2	1	5	1	2	[illegible]	3	3	2	0	2	1	0	0	0	3	96
Uterus	220	–	6	5	5	12	1	4	2	2	3	1	1	1	0	0	1	2	1	1	0	1	2	–	0	1	52
Rectum	199	15	1	2	4	3	2	3	3	1	–	1	2	1	4	1	0	1	0	0	1	0	0	0	0	3	48
Pancreas	196	20	4	[illegible]	4	[illegible]	4	1	[illegible]	–	1	3	8	1	0	0	0	0	0	0	1	0	0	0	0	0	48
Liver	186	18	3	[illegible]	2	0	5	4	1	0	[illegible]	0	–	0	0	0	0	0	0	0	0	0	0	0	0	1	38
Ovary	173	–	1	1	2	9	3	0	1	0	0	0	0	1	0	0	–	1	0	2	0	1	0	–	0	0	22
Bil system	172	6	3	2	1	2	[illegible]	3	0	0	4	0	0	[illegible]	–	0	0	1	0	0	0	1	0	0	0	1	28
Urinary bladder + urethra	149	11	6	1	1	2	1	2	1	1	1	0	0	–	2	2	1	0	0	3	0	0	0	1	0	0	36
Glioma	118		0	0	1	0	1	0	1	0	0	1	0	0	1	0	1	1	0	0	0	–	0	0	0	0	9
Small intest	113	13	8	4	3	[illegible]	1	6	1	3	1	–	0	0	0	0	0	0	1	1	0	1	1	0	0	1	47
Oesophagus	8	7	0	0	[illegible]	0		–	1	0	0	1	0	0	0	0	0	0	–	0	0	0	0	1	0	3	19
Skin	69	5	0	1		4	6	0	0	0	1	0	0	2	0	–	0	0	0	0	0	0	0	1	1	0	24
Thyroid	54	3	5	0	1	1	3	0	2	0	1	0	0	0	1	0	1	–	0	1	0	1	0	0	0	0	20
Total		249	115	96	95	91	91	89	49	47	44	44	37	37	27	22	20	19	15	14	9	9	7	6	[illegible]	47	1278

In malignant lymphoma are included also leucosis and multiple myeloma

with double cancers 73 8 years in those with triple cancers and 77 6 years in those with quadruple cancers In the entire autopsy series 51 0 per cent were men 52 4 per cent of the subjects with single tumours and 65 6 per cent of those with multiple malignant tumours were men This preponderance of men was due to the high frequency of microscopically demonstrable prostatic cancer

In Table 2 are listed the number of various malignant tumours and their combination with multiple tumours The percentage figures are higher than those in Table 1 because tumours and not individuals are listed and besides in cases with double cancer both organs are listed in cases with triple cancer the three organs are listed and in cases with quadruple cancer the four organs are listed

Table 3 gives the locations (or types) and numbers of other cancers among the most frequent primary tumours (> 50 cases) Consequently the figures of total number are lower than those in Table 2

DISCUSSION

The occurrence of multiple malignant tumours in one and the same patient was first described in 1869 by *Billroth* and was published as a curiosity The cases published at the turn of the century were also described as rarities but in 1932 *Warren & Gates* whrote that Only that person whose experience with malignant disease is limited is now thrilled by encountering a case Multiple malignant tumours are thus not so rare as formerly believed and must be considered in clinical practice The majority of the many publications on this subjects are based on single cases on autopsy series clinical series or mixed series or on compilations of literature cases

The series by *Mersheimer et al* (1964) who collected data from the Connecticut Tumour Registry represent a community analysis They found a 3 2 per cent incidence of multiple malignancy and their series probably represent one of the best available but they have many cases of multicentric cancer There are however recent studies (e g *Feremans* 1966 who described 1 004 cases diagnosed during 40 years) where nothing is said about the basic population or the autopsy frequency

The only reliable method to estimate the true frequency of multiple cancer is however within a defined population to perform thorough autopsies on subjects of whom detailed clinical records on previous diseases are available *Lombard et al* (1946) among others therefore omitted from their series patients who were alive because they found that the second tumour was detected at autopsy in half the cases (except in those with skin cancer) Too many authors have not payed due respect to this fact

The criteria of multiple cancers vary from author to author The most widely accepted criteria appear to be those used by *Warren & Gates* (1932) who state that the tumours should be histologically ma

lignant they should be separate and they should not be metastases In the present investigation we used an additional criterion namely that the tumours shouud be situated in different organs This was done because it is difficult or impossible to decide whether more than one tumour in a given organ should be regarded as multifocal primary tumour or metastasis As to primary liver cancer multifocal origin is probably common (*Elias* 1960) When the liver is exposed to a carcino genic agent it may be assumed that the entire organ is affected In the event of multiple foci in the liver it is however not possible to decide whether they should be regarded as multiple tumours or metastases This also applies to tumours of the urinary tract and of the colon and also to primarily generalized tumours such as the malignant lympho mas *Moertel* (1964)found the rate of occurrence of multiple primary malignant neoplasms to be 5 1 per cent but only 2 8 per cent if multi centric tumours of the same organs or tissues were excluded Only in 3 cases in our material were multiple tumours in paired organs not re garded as *one* tumour *vi* one case of bilateral mammary cancer where an adenocarcinoma appeared in the left breast 7 years after radical treatment of a carcinoma simplex on the right side (and without meta stases from any of the tumours at autopsy 3 years later) one case of bilateral bronchial carcinoma where one tumour was an oat cell carcin oma the other an adenocarcinoma one was a case of an adenocarcin oma of the left renal pelvis and a carcinoma of transitional cell type in the right ureter

Basal cell carcinomas were ignored firstly because of the benign nature of this lesion and secondly because it is difficult to ascertain the frequency of previous basal cell carcinoma in an autopsy series This difficulty also applies to squamous epithelial cancer of the skin In our series such tumours had doubtless occurred without being noted in the hospital records or so long ago that they were not covered by the files of the Department of Pathology It is also known that squamous epithe lial cancer is more common in clinical than in autopsy series (*Malmio* 1959)

Many of the tumours here designated as metachronous have in fact been synchronous since the second tumour(s) has been diagnosed clin ically or at post mortem shortly after radical treatment of the first tumour Since we know no safe method to decide whether a later diag nosed tumour was present at the time when the first tumour was cured the situation at autopsy was used

The frequency of multiple malignant tumours given in the literature varies widely (Table 4)

The frequency varies between 0 5 to 11 7 per cent (present material) This variation may be due to different factors Different authors use different criteria for multiple cases The series probably differ in age distribution Clinical series except those dealing only with skin cancers (see *Malmio* 1959) of course show a lower frequency and in the

autopsy series the type of hospital and the methods of autopsy (e g routine histologic examination of the prostate) and of selection of cases for autopsy will influence the results

TABLE 4

Frequency of Multiple Malignant Tumours in Autopsy and Clinical Materials

	Authors	Year	Total no investigated	No of malign tumours	No of multiple tumours	Mult malign tumours in %
Autopsy series						
1	Feilchenfeld	1901	5 022	507	10	2 0
2	Pedlich	1907	–	496	10	2 0
3	Cade	1915	–	4 219	32	0 8
4	*Ha bitz*	1916	2 613	524	16	3 1
5	Rau	1922	10 393	1 132	17	1 5
6	*Medi edjew*	1924	4 638	470	20	4 3
7	Junghanns	1929	26 043	4 219	19	0 5
8	Coriainowa & Schabad	19 0	6 652	1 233	23	1 9
9	Brandt & Jekabson	1930	14 893	2 083	11	0 5
10	Muller	1930	5 012	1 121	19	1 7
11	Hanlon	1931	3 000	950	38	3 9
12	Warren & Gates	1932	–	1 078	40	3 7
13	Burke	1936	2 033	583	46	7 8
14	Austin	19 8	8 124	887	24	2 7
15	Kirshbaum & Shively	1938	10 870	1 411	25	1 8
16	Hellendall	1943	–	685	30	4 3
17	Warren & Ehrenreich	1944	–	2 829	194	6 8
18	Albrecht	1951	31 630	6 188	203	3 3
19	Fried	1958	4 800	1 514	24	1 6
20	Rae	1958	2 130	711	60	8 4
21	Moertel et al	1961	–	37 580	1 909	5 1
22	Thoma	1964	2 346	–	–	4 3
23	Present series	1968	11 100	4 895	572	11 7
Clinical series						
1	Hurt & Broders	1932	–	2 124	71	3 3
2	Schreiner & Wehr	1934	–	11 212	07	2 7
3	Desaive et al	1939	–	3 115	36	1 2
4	Mider et al	1952	–	3 996	179	4 5
5	Wallace	1957	–	3 006	134	4 5
6	Malmi	1959	–	27 712	630	2 3
7	Polk et al	1964	–	1 141	121	10 6

See *Malmio* (1959)

The highest frequencies of multiple cancer described in the literature are those reported by *Polk et al* (1964) 10 6 per cent basal cell carcinoma included *Rae* (1958) 8 4 per cent microscopically demonstrable carcinoma of the prostate included and by *Burke* (1936) 7 8 per cent basal cell carcinoma included The high frequency reported by *Polk et al* (1964) is explained by the fact that they only studied the frequency of multiple cancers associated with carcinomas of the colon and of the rectum Since these carcinomas have a relatively good prognosis the

risk of developing a next tumour is greater than in many other tumours. To this must be added that basal cell carcinomas constituted more than one third of their multiple cases. They found that not less than one third of the patients who survived their first cancer for 5 years developed a new cancer. As mentioned, our criteria for multiple cancer are more rigorous in that multiple foci in a single or in paired organs were regarded as one tumour. Even if latent prostatic cancer were not included in our series, the frequency of multiple tumours would still be as high as 8.8 per cent.

The high frequency of malignancy, 44.1 per cent, among the autopsied subjects is a result of selection. In an attempt to ascertain the extent of the selection, the records of all the 2,224 subjects who had died in 1965 in Malmö were analysed. Out of these, 1,432 (64.4 per cent) were autopsied at the Department of Pathology and 752 malignant tumours were diagnosed in 645 subjects, which is 45.0 per cent of those autopsied. Among the 792 not autopsied here, 397 were autopsied at the Department of Forensic Medicine. Among these (many of them were young subjects) 33 malignant tumours were found. As regards the remaining subjects not autopsied, death certificates were issued by general practitioners in private practice. By studying the death certificates and the files of the Department of Pathology it was found that 21 tumours had been diagnosed in these patients. An autopsy would of course have revealed a number of cancers not diagnosed *intra vitam*. Since the 54 tumours represent less than 10 per cent of those in autopsied subjects, it would be better to correlate the autopsy material to the total number of deaths in the area. Since such studies are difficult and demand complicated statistical methods, we intend to return to the problem in a later publication.

It is seen from Table 1 that approx. 10 per cent of all cancer cases had a second tumour, 10 per cent of those had a third tumour and 10 per cent of those had a fourth tumour. We are at the moment not able to explain this regularity (which may be incidental). There is no absolute correlation between the frequency of multiple cancer and mean age at death (Table 2). As a rule (if we exclude tumours constituting only a few cases) low mean age at death is associated with a low frequency of multiple tumours. This explains the low frequency of multiple tumours in cases of glioma. In cases of mesothelioma the frequency is very low despite the high mean age at death. This is probably due to the unwillingness of diagnosing a mesothelioma when the subject has another tumour which possibly could be responsible for the growth in the serous cavities. The high frequencies of multiple cancer associated with carcinomas of the prostate, kidneys and small intestines respectively depend on the large number of microscopically diagnosed prostatic tumours, that in the kidney cortical adenomas with a diameter of more than 2 cm were regarded as carcinoma and

that many small carcinoids of the small intestine were diagnosed incidentally at autopsy.

In Table 3 the sites and types of primary cancers are given in the left column in order of decreasing frequency. When locations and types of the other tumour(s) are given in the same way it is seen that the distribution of multiple malignant tumours among the various organs of the body is approximately the same as that of solitary tumours.

A question often discussed in the literature is whether the risk of development of a second tumour in a patient with one malignant tumour is greater than the risk of development of an initial malignant tumour in a given population. *Peller* (1941) for instance believed that once a tumour had developed it would prevent immunologically the development of further tumours. The frequency found in some series (*Muller* 1930, *Watson* 1953) and also in the present one lends no support to such an assumption. Some authors (*Warren & Gates* 1932, *Bugher* 1934, *Warren & Ehrenreich* 1944) believe that the frequency of second and third tumours is higher than that ascribable to chance.

Watson (1953) followed up 16 626 cancer patients for whom the man years exposure to the risk of developing a second cancer was computed and the author compared this risk with the risk which according to the figures for the normal U.S. population is involved. He was unable to demonstrate any evidence of a constitutional tendency to develop a second tumour, or of an immunity following the first.

Malmio (1959), who includes multicentric cancer, was not able to show that chances of contracting a new tumour were higher in patients with a single cancer than chances of such development in the population at large. He found, however, like *Finhorn & Jacobsen* (1964) that multiple tumours were more common among young subjects than among elderly ones. Our series does not seem to support this last finding. Since multiple tumours in the same or in paired organs were excluded in our material (with the exception of three cases) it has not been possible to study whether the risk of development of a second tumour in an organ already harbouring a tumour is greater than the risk of development of an initial tumour in the same organ. The existence of such a risk is, however, apparently well documented (*Lund* 1933, *Moertel & Soule* 1957, *Qualheim & Gall* 1957, *Moertel et al* 1961).

It is a well known fact that patients with multiple tumours survive longer than patients with only one tumour (*Warren & Gates* 1932, *Warren & Ehrenreich* 1944, *Lombard et al* 1946, *Malmio* 1959) but this seems only to mean that the patients are apt to develop a new tumour because of their long survival.

It would be interesting to know how often multiple tumours are of interrelated origin. Theoretically, one might imagine that certain tumours are hormone dependent and thereby apt to occur in association with one another. *Moertel* (1964) was unable to demonstrate any such

association It is however accepted by *Fried* (1958) and *Hersheimer et al* (1961) who suggest that it may possibly be related to an endocrinologic factor

This problem is extremely difficult to analyse and we do not think that it is possible on the basis of our large series to give any definite answers It is seen from Table 3 that 22 other tumours were found among the 173 cases of ovarian malignancy Nine of these 22 i e 41 per cent were cancer of the breast while breast cancer is responsible for less than 20 per cent of all female cancer If we however look at the 478 cases of breast cancer it is seen that only 9 per cent of the other tumours are found in the ovaries which are responsible for 7 per cent of female malignancies This seems to indicate that subjects with ovarian cancer are apt to develop breast tumour but not *vice versa* The explanation might be that the serum estrogen level is high even in cases of non hormone producing ovarian tumours

It is important to remember as pointed out by *Clemmesen* (1965) that malignant diseases with a high cure rate when occurring early in life will offer a possibility for the development of a second cancer greater than that of other cancers To this comes the variation in risk with age for cancers at different sites

SUMMARY

In an investigation of the frequency of multiple tumours in a defined population (Malmo 250 000 inhabitants) where approx 60 per cent of all subjects are autopsied at death the following findings were obtained

1 Out of 11 098 patients examined *post mortem* 4 895 (44 1 per cent) had or had had 5 523 malignant tumours

2 Multiple tumours syn- or metachronous at autopsy were found in 572 subjects (11 7 per cent)

3 The types and locations of multiple malignant tumours were roughly the same as those of solitary tumours

4 The frequency of multiple tumours was with certain exceptions which are explained associated to mean age at death

5 The frequency of multiple tumours increased with duration of survival after treatment

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THE TWO TYPES OF A CELLS IN THE ISLETS OF LANGERHANS OF NORMAL AND SYNTHALIN TREATED GUINEA PIGS

By

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CLAES HELLERSTROM

Received 2 xii 68

The morphological and functional characterization of the various cell types of the islets of Langerhans has been a matter of growing interest during recent years It has been shown that the islets of most species studied up to now contain in addition to the insulin producing B cells also A_1 cells displaying an argyrophil reaction with a modified Davenport technique and A_2 cells which lack this property (*Hellerstrom & Hellman* 1960 *Hellman & Hellerstrom* 1969) Some authors claim that the A_1 cell and the islet D cell of *Bloom* (1931) and *Thomas* (1937) are identical (*Epple* 1964 *Solcia & Sampietro* 1965 *Fujita* 1964 1968 *Tillbach* 1968), whereas others do not accept this view without reservation (*Hellerstrom & Asplund* 1966 *Bjorkman et al* 1966 *Östberg et al* 1966) This controversy seems now to depend mainly on the observation that in the islets of foetuses and certain lower vertebrates the classical granule stains fail to differentiate between the granular D cells and the agranular islet cells (*Bjorkman et al* 1966 *Östberg et al* 1966 *Epple* 1968)

While it seems well established that glucagon is elaborated by the A_2 cells (*Unger et al* 1967) the biological significance of the A_1 cells is still an enigma It is not even known with certainty whether the latter cell type should be regarded as functionally independent or indeed if it exhibits any secretory activity at all Evidence against the view that the A_1 cell is a particular functional stage of another islet cell type has been derived from its specific histological and histochemical staining characteristics and its reactions to various experimental procedures (*cf Hellerstrom et al* 1964 *Hellman & Hellerstrom* 1969) On the other hand mixed forms of A_1 cells and A_2 cells have been reported in some studies (*Bjorkman et al* 1966 *Bjorkman & Hellman*

This investigation was supported by grants from the Swedish Medical Research Council (B69-12X-109 05 B69 12X-2297-02) the Medical Faculty of the University of Uppsala and the United States Public Health Service (AM 12335) The skilled technical assistance of Miss Anna Britta Andersson is gratefully acknowledged

1964 *Erbengi* 1964) The present investigation was carried out to eval uate further the specific properties of the two types of A cells by ana lysing these cells after administration of an A cytotoxic agent Synth i lin A to guinea pigs

MATERIAL AND METHODS

Altogether 75 male guinea pigs weighing 200-700 g were used The animals had free access to water and a diet consisting of hay root vegetables and pellets Syn thalin A (decamethylenediguanidine dihydrochloride L Light & Co Ltd C ln brook England) was dissolved in sterile isotonic saline to a concentration of 1 mg/ ml and injected subcutaneously The solution was given either as a single injec tion (3 35 mg/kg b w) or as one daily injection (2 mg/kg b w) for three sub sequent days (Table 1) The animals were killed by a blow t the head and ex sanguinated 44-77 hours after the first injection Control animals were treated similarly except that injections consisted of sterile isotonic saline only

At various intervals during the experimental period blood samples were ob tained from the ears and at the time of killing from the severed neck vessels The blood glucose concentration was determined with the glucose oxidase method as described by *Hjelm & de Verdier* (1963) After death the pancreas was rapidly re moved and small pieces of tissue were either frozen at —70 C in isopentane or fixed at room temperature in Zenker form l solution (*La arus & Voll* 19?) The fixed tissue specimens were dehydrated and embedded in paraffin or Epon 812 (*Luft* 1961) Cryostat microtome sections 10 μ thick were obtained fr m the frozen blocks and immediately studied in dark field illumination Sections 4 or 7 μ thick were cut from the paraffin embedded material and either silver impregnated (*Hel l rström & Hellman* 1960) or granule stained with chrome haematoxylin (*Bencosme* 1952) or aldehyde fuchsin In both these latter cases the counter stain was pon ceau fuchsin In addition some sections were stained with phosphotungstic acid haematoxylin (PTAH *Lillie* 1965) The Epon-embedded tissue was cut into sections 0 5-1 μ thick which were silver impregnated or stained with aldehyde fuchsin ponceau fuchsin after removal of the plastic with an alcoholic NaOH solution (*Van & Europa* 1965) Classification of the two types of A cells was accomplished by photography of silver impregnated islets followed by removal of the silver and re staining with one of the granule stains (*Hellerström & Hellman* 1960)

TABLE 1

Dosage of Synthalin A and Survival Rate in the Different Groups of Experimental Animals

Number of animals	Synthalin A (mg per kg b w at each injection)	Number of injections	Number of animals surviving after 3 days
14	2 0	3	14 (100 %)
22	3 0	1	13 (59 %)
14	3 5	1	6 (43 %)

The content of refractile silver white granules in the cytoplasm of the A_2-cells was graded semiquantitatively in dark field illumination of the cryostat micro tome sections the microscopist being confronted with "unknown sections The procedure was applied to pancreatic material fr m 7 control animals and 9 animals which had received daily injections of Synthalin A (2 mg/kg b w) during 3 con secutive days All of these animals had been killed 24 hours after the last injection

Quantitative histological analyses were performed in 8 Synthalin treated guinea pigs and 9 control The treated animals had received the drug in different dosage according to Table 1 and were selected among those animals which had clear mor



Figs 4–5

Fig 4 Detail of a vacuolated A_1 cell in a thin chrome haematoxylin ponceau fuchsin stained section from Epon embedded pancreatic material. Some (acidophil) granules may be distinguished in the remaining cytoplasm (arrows) × 2000

Fig 5 Mitotic figure in an islet cell which may represent a vacuolated A_1 cell. No distinct (acidophil) cytoplasmic granules could be identified. Technique as in Fig. 4 × 2000

Synthalin injected animals contained obviously enlarged nuclei with decreased density of the chromatin net. Pycnotic nuclei were seldom seen in the injured cells. In addition mitotic figures were recorded in a few islet cells which however could not be classified according to type (Fig. 5).

TABLE 2

Effects of Synthalin A Treatment on the Percentage Contribution of A_1 Cells to the Total Islet Volume and on the Mean Nuclear Size of the Different Islet Cell Types. Means ± S.E.M.

Animal group	A_1 cells %	Nuclear size (Arbitrary units) A_1 cells	A cells	B cells
Controls (9)	10.4 ± 0.9	1.1[illegible] ± 0.05	1.[illegible]9 ± 0.03	1.17 ± 0.04
Synthalin A treated (8)	8.8 ± 0.6	1.09 ± 0.05	1.65 ± 0.10	1.11 ± 0.05

The quantitative histological methods were applied to those Synthalin treated animals in which there were A_1 cells displaying clear morphological changes. As can be seen in Table 2 the percentage contribution of A_1 cells to the islet volume in the treated animals did not differ

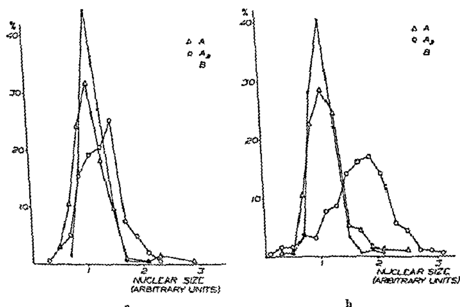


Fig 6

a) Size frequency distribution of the nuclear section areas from the various islet cell types in normal guinea pigs b) A corresponding plot from guinea pigs treated with Synthalin A It is evident that in these animals the peak of the A -cell curve is displaced to the right and that the curve is considerably broadened

from that in the control animals ($t = 1\ 32$ $P>0\ 05$) Likewise there was no difference between the two groups in the nuclear size of the A_1 cells ($t = 0\ 44$ $P>0\ 05$) or the B cells ($t = 1\ 26$ $P>0\ 05$) By con trast the nuclear size of the A cells was significantly increased in the Synthalin injected animals ($t = 3\ 71$ $P<0\ 01$) When the nuclei of the various islet cell types were classified according to size the frequency curves on the whole showed symmetrical distributions (Fig 6) In the Synthalin treated animals the peak of the A cell curve was dis placed towards higher values and the base of the curve was consider ably broadened

DISCUSSION

It emerged from the present study that all those cells which exhibited cytoplasmic vacuoles and degranulation after administration of Syn thalin A should be classified as A cells In addition quantitative ana lyses showed that changes in nuclear size were confined to this cell type A lack of effect on the A cells was further indicated by the find ing that these cells contributed equally to the islet volume in the two animal groups These observations confirm and extend previous reports indicating a selective effect of Synthalin A on the pancreatic islet A-cells (*Davis* 1952 *Korp & LeCompte* 1955 *Creutzfeldt & Tecklenborg* 1955 *Munger* 1962 *Solcia & Sampietro* 1965) Although the present

data support the view that the A_1 and A cells are physiologically separate entities it cannot as yet be excluded that these cell types represent different stages in a common functional cycle It has been reported for example that islet B cells with a relatively low rate of insulin secretion are much more susceptible to the toxic action of alloxan than those with a high functional activity (*Lazarus & Volk* 1962)

Little is known regarding the mode of action of Synthalin A on the islet A cells It has been suggested that administration of the drug brings about an increased functional demand on these cells some of which may be exhausted as evidenced by the presence of cytoplasmic vacuoles (*Creutzfeldt* 1960) A more direct cytotoxic effect of Synthalin A has also been proposed and the hypoglycaemic effect of the drug was then assumed to reflect a deficiency of glucagon (*Holt et al* 1955) In the present study the A cells displayed both degranulation and an increased mean nuclear size whereas pycnotic cell nuclei were rare even in severely vacuolated cells These observations seem to corroborate the view proposed by *Hultquist* (1959) and *Creutzfeldt* (1960) that the A cells respond to Synthalin A with increased functional activity and sometimes exhaustion rather than cytotoxic degeneration

In addition to Synthalin A and some other guanidine derivatives (*Creutzfeldt & Moench* 1958) certain salts of heavy metals particularly cobaltous chloride have been found to be injurious to some A type cells of guinea pig islets (*van Campenhout & Cornelis* 1951) The lesions brought about by these agents appear morphologically very similar to those observed after administration of Synthalin A Moreover the effect of cobaltous chloride also seems to be confined to the glucagon producing A cells (*Petersson et al* 1962) This is further supported by the recent observation of a marked increase of the plasma glucagon activity after administration of this compound to rats (*Lochner et al* 1964) Whether there is a common mechanism for the effect of guanidine derivatives and certain metal salts on the A cells remains a matter of speculation It is worthy of note that in the guinea pig cobaltous chloride does not seem to decrease the blood sugar level (*Creutzfeldt & Schmidt* 1954) whereas Synthalin A brings about a characteristic hypoglycaemic state

SUMMARY

Light microscopical analyses of the two types of pancreatic A cells were performed after subcutaneous administration of Synthalin A to guinea pigs In about 50 per cent of the treated animals a moderate number of islet cells showed a conspicuous cytoplasmic vacuolization By analysing pancreatic sections which had first been silver impregnated and later after removal of the silver restained with one of the granule stains it was found that the affected cells should be classified as A cells no morphological changes in the A_1 or B cells were recorded In addition the A cells showed an increased mean nuclear size

and a marked degranulation after Synthalin A The results further support the view that the A_1 and A_2-cells are physiologically separate cell types

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FINE STRUCTURE OF ARTERIOSCLEROSIS INDUCED IN RABBIT AORTA BY EPINEPHRINE AND THYROXINE

By

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Received 5 xii 68

Intravenous injection of epinephrine in the rabbit produces arteriosclerotic lesions which are considerably aggravated by simultaneous administration of L thyroxine An increase in the mucopolysaccharide content and in the uptake of ^{35}S with unchanged hydroxyproline content was found in such conditions by *Lorenzen* (1959 1961) The technique of electron microscopy of mucopolysaccharide molecules is insufficiently developed The present electron microscopical study was intended to reveal epinephrine induced lesions in the rabbit aorta The experimental procedure was similar to that used by *Lorenzen* (1959 1961)

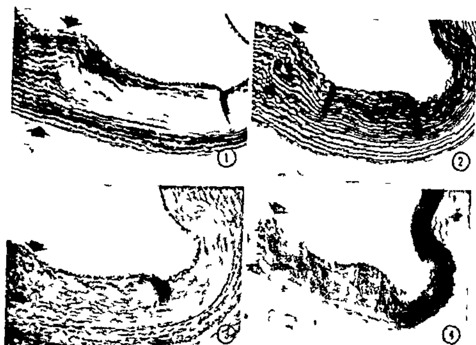
MATERIAL AND METHODS

Through one week three white male rabbits (body weight approximately 2 kg) had daily intravenous injections of epinephrine During the first five days the dose was increased gradually from 0.025 mg/kg to 0.050 mg/kg L-thyroxine 0.05 mg/kg daily was injected subcutaneously in an aqueous suspension through the same period (*Lorenzen* 1961) After one week the rabbits were killed by intravenous injection of 200 mg of Nembutal® The thoracic aorta was removed. The intimal surface exhibited three kinds of gross changes i e grossly normal nodular and paper like areas Specimens were removed from these areas and fixed in an ice-cold 1 per cent osmic acid solution of veronal acetate buffer pH 7.2 After dehydration in graded alcohols specimens were stained with a 1 per cent phosphotungstic acid solution in absolute alcohol and embedded in prepolymerized methacrylate Ultra thin sections (about 500 A) were cut by an LKB ultramicrotome and observed by a Philips 100 B electron microscope at 60 kV For histological study 3μ thick sections were cut of the same specimens by the same ultramicrotome After removal of the methacrylate by acetone the thick sections were stained by haematoxylin eosin resorcin fuchsin toluidine blue and calcium red (*McGee Russell* 1955)

RESULTS

Gross findings The inner surface of the thoracic aorta of all animals exhibited nodules and plaques The nodules were of about five milli

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Figs 1 2 3 and 4

Specimens from a paper like area Arrows indicate the borderlines between the plaques (right) and the grossly normal areas (left) 1 Toluidine blue staining 2 Acid orcein staining 3 Haematoxylin eosin staining 4 Calcium red staining Fig 1 shows unstained elastic lamellae The medium dark areas between the elastic lamellae are the metachromatically stained extracellular spaces In Fig 2 the elastic lamellae of the diseased area show no wavy appearance Fig 3 shows very few nuclei in the media of the diseased area and numerous spindle shaped nuclei out side the lesion Fig 4 Calcified areas are stained by calcium red

metres in diameter oval cloudy slightly elevated and well demarcated against the surrounding smooth surfaces The plaques were irregularly oval with a long axis of one or two centimetres not elevated and with a paper like wrinkled surface

Histological findings After toluidine blue staining all smooth muscle cells of the grossly normal areas of the aortic wall appeared blue stained The spaces between these smooth muscle cells and the elastic lamellae were stained metachromatically (Fig 1) No changes were found in the endothelial cells nor in the elastic lamellae In nodular areas with a smooth surface and areas with a paper like wrinkled surface the smooth muscle cells appeared like those of the grossly normal areas However the elastic lamellae were arranged straight and parallel and each of them revealed thinnings and fragmentations (Fig 2)

The endothelial cells were flat and showed desquamation in certain areas These changes were more severe in the paper like wrinkled areas than in the nodular areas Very few nuclei were found in certain lesions which were surrounded by numerous spindle shaped nuclei (Fig 3) In



Fig 5

A grossly normal area of aortic wall Two smooth muscle cells in the centre of the photograph are oval and contain nucleus (N) mitochondria (M) and vacuoles (V) Arrows indicate the connections of these cells to elastic fibres In the right side of the photograph a smooth muscle cell shows an exceptionally dense cytoplasm Dense thread like material is seen on the surfaces of the elastic lamellae (EL) In the space between the lamella and the smooth muscle cells a dense granular material (G) and collagen like fibrils (C) can be seen × 8 400

the paper like area calcium deposits were demonstrated in the media by calcium red staining (Fig 4)

Electron microscopical findings In the grossly normal areas some smooth muscle cells of the inner and middle layers of the media were oval with vacuoles (Fig 5) or cystic cytoplasmic protrusions containing fine granular or thread like material (Fig 6) Others showed a dense cytoplasm consisting of myofilaments vacuoles and mitochondria An attachment of smooth muscle cells to elastic lamellae was seen in a few areas The elastic lamellae showed no internal changes but the surfaces of the lamellae and their branches showed dense fine granular material or thread like figures (Fig 7) The spaces between the smooth muscle cells and the elastic lamellae were filled with dense fine granular material dense elastic filaments and collagen like fibres the latter



Fig 6

A grossly normal area of aortic wall Note large cystic protrusions of the sarco plasm of a smooth muscle cell (CP) Arrow indicates the connection between these protrusions and degenerated elastic fibres The elastic lamella (EL) and the elastic branch (E) show dense fine granular material on their surfaces (G) This material is also seen in the spaces between the elastic fibres and the cystic protrusions of the smooth muscle cells There are collagen like fibril (C) in the same space
× 14 800

showing no transverse banding (Fig 7) The outer parts of the media showed changes similar to those of the inner and middle layers The endothelial cells contained nuclei mitochondria and vacuoles and showed clear intercellular connections However the elastic fibres anchoring the endothelial cells appeared granular like the fibres between the lamellae (Fig 8)

In the nodular and paper like areas the endothelial cells were flat or oval containing a nucleus while other cell organelles were indistinguishable (Fig 9) The connections between two neighbouring endothelial cells were distinct and the anchoring elastic fibres were granular similar to those of the apparently normal area (Fig 9) The smooth muscle cells below the endothelial cells contained dense granu



Fig 7

A grossly normal area of aortic wall Between the elastic lamellae (EL) a smooth muscle cell containing vacuoles mitochondria and myofilaments is shown It has direct connections to dense elastic fibres (arrows) Dense fine granular material is found on the surface of the elastic lamellae (framed arrows) In the spaces between the elastic lamellae and the smooth muscle cell note numerous collagen fibres (C) dense elastic fibres (DE) and dense granular material (G) × 8 5'0

lar material but myofilaments or other cell organelles could not be identified Between the elastic lamellae of the media the smooth muscle cells showed compact masses of myofilaments The elastic *lamellae were straight and parallel (Fig 9) The spaces between the* elastic lamellae and smooth muscle cells were filled with collagen like fibrils and degenerated elastic fibrils The outer area of the media contained spindle shaped fibroblasts (Fig 10) In the paper like area calcium precipitates were found as extremely dense granular masses and needle like crystals on the surfaces of the elastic fibres (Fig 11)

DISCUSSION

The main alterations in rabbit arteriosclerosis induced by epinephrine plus thyroxine occurred in the smooth muscle cells and the elastic fib



Fig 8

An endothelial cell of a grossly normal area Note nucleus (N) mitochondria (MI) and small vacuoles (V) The anchorings under the cell appear destroyed and dense elastic fibres (DE) are seen Note connections to the adjacent endothelial cells (arrows) × 1… 600

res of the aorta very much like the lesions seen in arteriosclerosis of man (*Geer et al* 1961 *Kawase* 1963 *Daoud et al* 1964 *Haust et al* 1967) and domestic animals (*Kawase* 1963 *Still* 1964 *Dahme* 1965 *Geer* 1965 *Parker et al* 1966 *Knieriem* 1967) In the present study there were no foam cells with fat droplets like those seen in human atherosclerosis but the smooth muscle cells revealed structural changes such as cystic protrusions of the sarcoplasm vacuole formation and condensation of the myofilaments

As to the granular or thread like material of the extracellular spaces *Haust et al* (1965) using material from human atherosclerosis and electron microscopic immunohistochemical technique demonstrated the presence of fibrin *Haust et al* (1967) believed that the lipid components of human atherosclerosis coexisted with degenerated elastic fibres remnants of fibrin or detached basement membrane of smooth muscle cells In the present study a minor part of the dense thread like



Fig 9

Paper like aortic area The endothelial cells (E) are flat showing no clear cell organelles or anchorings There are connections with the neighbouring endothelial cells (arrows) The smooth muscle cells (M) reveal dense masses of myofilaments and a complete loss of connections with elastic lamellae The elastic lamellae (EL) are straight Dense elastic fibres (DE) and collagen like fibres (C) are located in the extracellular compartments × 8 500

material along the elastic fibres and in the area of degenerated smooth muscle cells may represent degenerated elastic material and myofilaments However most of the granular and dense thread like material could not be identified as such structures nor as remnants of fibrin or all mixed together The numerous collagen like fibrils in the extra cellular spaces showing no distinct transverse bands were parallelled by increased total contents of hydroxyproline in the biochemical studies of *Loren en* (1959 1961)

Degeneration of elastic fibres may give rise to disanchoring of the endothelial cells and smooth muscle cells (*Bierring et al* 1963) It may cause desquamation of the endothelial cell layer and together with changes of smooth muscle cells loss of waviness of elastic lamellae The areas where the dense thread like and granular material were found

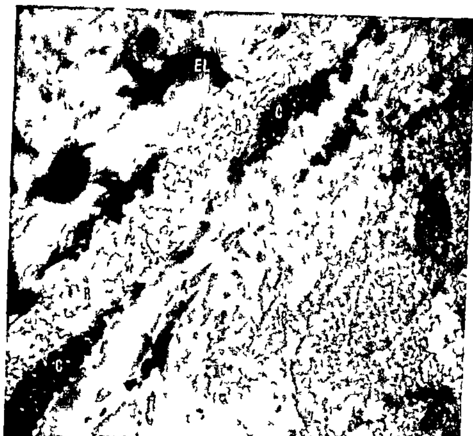


Fig 10

Outer part of the media in a paper like area Cells containing endoplasmic reticulum (R) probably fibroblasts are seen between the elastic lamellae (El) In the extracellular space there are collagen like fibrin (C) × 27 600

were the same as those staining metachromatically However the dense material could not be identified with certainty as the acid mucopolysaccharides demonstrated earlier by histochemical and biochemical methods

The same type of calcification demonstrated on the surfaces of the elastic fibres has previously been found after three weeks in epinephrine influenced rabbit aorta by *Lu et al* (1965)

In atherosclerosis of man and domestic animals the pathogenetic role of the endothelial cells has been discussed by *Dahme* (1965) *Geer et al* (1961) *Geer* (1965) and *Still et al* (1964) In the present study on arteriosclerosis the endothelial cell changes seemed to be less significant than those in atherosclerosis



Fig 11

Calcification of elastic lamellae (EL) Extremely dense granular precipitates (GR) and needle like crystals (CR) are seen This area showed intense red histochemical stainability with calcium red × 85 800

SUMMARY

Experimental arteriosclerosis induced in the rabbit by epinephrine plus thyroxine was studied by electron microscopy and ordinary light micro scopy The manifest changes were degeneration of elastic fibres cystic protrusions from and vacuoles in the sarcoplasm and condensation of *myofilaments of the smooth muscle cells Calcification of the* elastic fibres and degeneration of the endothelial cells were also found

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THE PULMONARY VASCULAR PATTERN IN IDIOPATHIC RESPIRATORY DISTRESS

A Micro Angiographic Study

By

Björn I Ivemark and Göran Wallgren

Received 13 xii 68

The Idiopathic Respiratory Distress Syndrome (IRDS) is associated with the formation of air space lining membranes made up of blood protein (5) This circumstance together with the recent observation that pulmonary ischaemia is a component in the clinical entity (1 2) has inspired a steadily increasing interest in the structural appearance of the microcirculation of the lungs in IRDS

Micro angiographic studies of the pulmonary circulation in the perinatal period illustrate the pulmonary vascular bed down to the capillary level (18) and is a suitable tool for this purpose in IRDS studies (9 12) It has been suggested (9) that the precapillary and capillary filling is reduced in pulmonary microradiograms from IRDS infants

The post mortem lung of infants dying from IRDS is almost invariably collapsed due to terminal resuscitation efforts with oxygen In view of the fact that structural changes are known to occur in the vascular tree when the lung expands (17) and the vascular resistance is known to diminish when the collapsed lung is aerated (4) it would seem desirable to investigate lungs from infants with IRDS with a technique that permits the injection of an isotonic contrast medium into the pulmonary circulation when the organ has been adequately aerated or preferably is rhythmically ventilated during the injection This would serve the double purpose of making the IRDS lung more "normalized" with respect to the architecture of the vessel as well as promote the filling of the smaller vessels

The present investigation was undertaken in order to evaluate possi

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This investigation has been supported by grants from *The Swedish National Association against Heart and Chest Diseases Stiftelsen Therese och Johan Anderssons Minne* and *Stiftelsen Gustaf och Tyra Svenssons Minne*

MATERIAL

Autopsy specimens from 19 perinatal cases were used for the experiments *Five* pairs of lungs were *obtained* from non macerated *stillborn* infants (birth weight 1 910–4 060 grams) *six pairs* of lungs from *newborn* infants who died from extra pulmonary *causes and who had no evidence of* cardiovascular malformations (*birth weight* 1 140–3 150 grams survival time 10 minutes–4 days) *eight* pairs of lungs were *derived from* infants with *respiratory distress* in all of whom hyaline membranes were histological *demonstrable (birth weight 1 150–2 890* grams survival time 10–48 hours) *In all groups about half* of the specimens had been kept deep frozen at −20 during preparation for this study The *specimens were thawed at room temperature* At autopsy great care was taken not to cut into the lung parenchyma and the specimens used for the study were all intact In the last two groups the material was obtained less than 20 hours after death

RESULTS

Micro Angiography

When micro angiograms were studied without knowledge of the clinical data it was not possible to delineate specific vascular patterns for any one of the groups The only constant finding within all groups was that the filling increased with maturity as reflected in the birth weights

All specimens were found to be technically adequate The pulmonary artery and its branches including the arterioles were usually filled In some regions in all specimens there was also capillary filling In none of the specimens injected from the arterial side was there any filling of the pulmonary or bronchial veins *Aeration of the lung caused* a structural change of the vascular tree owing to the intermixing of air filled spaces *but respiratory movements during* contrast injection did not seem to enhance capillary filling nor was the presence of arterio venous shunts revealed

In view of findings by other workers in this field (9) a different pattern was expected in the various groups of cases Specifically we expected to find a poor outline of the pulmonary vasculature in the case of respiratory distress In fact this was not the case In the early phases of the experiment occasional instances of poor filling of the pulmonary arterioles were found in lungs from cases of respiratory distress but later similar instances were encountered in cases of still

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Figs 2–3

Fig 2 *Hyaline membrane disease* Micro angiograms (PA injection) from two specimens *without ventilation* A Birth weight 2 [illegible]00 g gestational age 36 weeks death at 24 hours *Poor filling of arterioles* and capillaries *in left lower lobe* × 12 B Birth weight 2 170 g gestational age 34 week death at 24 hours Good filling of arterioles and capillaries of right lower lobe × 12

Fig 3 *Hyaline membrane disease* *Micro* angiograms (PA injection) from two specimens *after ventilation* 30 minutes negative pressure (*see text*) A *Same* case as illustrated in Fig 2 A Left upper lobe was ventilated and shows the same appearance as the non ventilated left lower lobe (Fig 2 A) *with poor* filling × 12 B Same case as illustrated in Fig 2 *B* *Moderate filling* of left lower lobe ventilation × 12


2a
2b
3a
3b


5a
5b

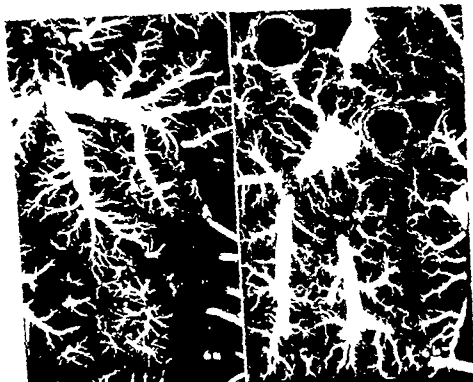


Fig 6

Micro angiography after injection into only pulmonary veins A Newborn birth weight 2 730 g gestational age 37 weeks death at 4 days Atresia of aortic arch Normal pattern × 12 B Hyaline membrane disease Birth weight 1 690 g gestational age 31 weeks death at 5 days Normal pattern including peribronchial venous plexa × 12

Figs 4–5

Fig 4 *Stillborn infants* Micro angiograms PA injection after ventilation (A) and without preceding ventilation (B) A Non macerated twin A birth weight 2 200 g donor in placental transfusion syndrome Good filling of arterioles and capillaries after negative pressure breathing × 12 B Non macerated stillborn birth weight 4 000 g tentorial tear Poor filling of capillaries and arterioles Injected without preceding ventilation × 12

Fig 5 Newborn infants with ut pulmonary disease Micro angiograms (PA injection) after ventilation (A) and without ventilation (B) A *Birth weight* 3 100 g gestational age 38 weeks age at death 24 hours Traumatic bilateral adrenal haemorrhage Right upper lobe was ventilated before micro angiography Poor filling of arterioles and capillaries × 12 B Same case as in A Non ventilated right lower lobe shows good filling × 12

birth and newborns without evidence of hyaline membrane disease The vascular pattern in the three groups is illustrated in Figs 2-6

With the technique used no arterio venous anastomoses were found The same number and type of anastomoses between the bronchial and pulmonary arteries is described by *Robertson* (18) were encountered in all three groups In a few cases in each group pulmonary venous injection was performed The venous micro angiograms produced did not show any difference between the groups (Fig 6)

Histology

The microscopic sections from the micro angiographed blocks confirmed that no ruptures had occurred during injection In four cases there was interstitial emphysema In serial sections no arterio venous communications could be demonstrated All specimens from cases of clinical respiratory distress showed the presence of hyaline membranes In no instance was there any inflammatory reaction The *lymphatics* of the lungs were studied systematically in all sections in view of the interest in the lymph flow of the lung in respiratory distress which recently has emerged (11 16 25) The perivenous lymphatics were markedly widened in all lungs with *hyaline membranes* This was also the case in the lobes of such lungs as had not been ventilated post mortem The lungs from newborn infants *without pulmonary disease* had negligible dilatation of the pulmonary lymphatics In one of these cases there was a difference between ventilated and non ventilated parenchyma inasmuch as the non aerated lobes showed moderate lymphatic dilatation while the aerated lobes did not show such dilatation a fact that might mean that ventilation may assist in drainage of pulmonary lymph The series of lungs from *stillborns* showed no dilatation of lymphatics regardless of whether or not the lobes had been ventilated post mortem

COMMENT

The histological examination of the lungs following micro angiography confirmed that the respiratory distress group contained only cases with non inflammatory hyaline membrane disease without aspiration the live newborns without IRDS showed no pulmonary changes except final interstitial haemorrhage and the stillborns had no appreciable pulmonary changes such as aspiration *etc* The four cases with experimentally induced emphysema belonged in the group of stillborn (3 cases) and newborn infants without IRDS

Although the series is small the vascular pattern of the lungs from the three groups seem worthy of a comparison The *microscopical* sections had the well known characteristics of pulmonary structure in respiratory distress The dilatation of pulmonary *lymphatics* in IRDS-lungs in man as described by *Wade Evans* (25) and *Lauweryns et al*

(10 11) and in lambs as described by *Normand et al* (16) was confirmed This lymph angiectasis was prominent in all such lungs and it was minimal or absent in the two other groups with one exception This was the lung from a live born without IRDS which had moderate dilatation of lymphatics in a lobe that had not been ventilated during the experiment but which had normal lymphatics of the aerated lobes The possibility of artifact seems to be remote since a) Rheomacrodex was used in the contrast medium instead of water and b) the remaining 9 specimens without hyaline membranes did not show lymphangiecta is It seems possible that decreased ventilation interferes with the drainage of lymph as suggested by *Schulz* (20) in a discussion of the electron microscopic findings of lymphangiectasis in lungs after extracorporeal circulation That a similar explanation should pertain to the constant finding of lymphangiectasis in the IRDS lungs seem less likely The cause of the—apparently experimentally irreversible—lymphangiectasis in the IRDS lungs still remains to be explained

Likewise the arterial pattern in lungs with hyaline membrane disease has been interpreted differently by several workers Some consider a premature dilatation of the pulmonary vessels the cause of respiratory distress (24) others consider the pulmonary arterial media to have normal thickness *i e* neither dilated nor constricted (8) and still others describe marked constriction (15 22) and in lambs even arteritis of the small arterioles (22)

However the vascular pattern as revealed by our micro angiography studies showed no appreciable difference between the groups At an initial stage of our experiments immature IRDS lungs showed areas with poor arteriolar and capillary filling This observation was conveyed to *Dr Lauweryns* (11) However as our study progressed it became evident that areas of such incomplete filling occurred in lungs from all groups particularly in immature lungs In essence then our conclusion is that there is no anatomical difference in the post mortem pulmonary vascular pattern in IRDS as compared with normal lungs of comparable gestational ages This statement includes arterial anastomoses which were of the same number and appearance as in normal lungs described by *Wagenvoort & Wagenvoort* (26) and *Robertson* (18)

Whether the differences in technique used in *Lauweryns* and our experiments can explain the discrepancy in the results is difficult to evaluate *Lauweryns* used a thick water suspension of barium sulphate (40 g/60 ml) compared to ours (7 5 per cent barium sulphate) in 6 per cent Rheomacrodex Our medium had a pH of 4 8 a value that should favour "vasoconstriction rather than dilatation The pulsatile flow that we used is perhaps comparable to his continuous flow in 2 *min increments* but might explain our better filling An additional and essential difference in experimental model is that our controls con

sisted of human lungs identically prepared while *Lauweryns* used lungs of lambs

Our main findings of anatomically normal arterial pulmonary vascular pattern in IRDS of course reflects the conditions post mortem It has no bearing on the previous existence of an *in vivo* vasoconstriction as the background of a hypoperfusion in IRDS (1, 2 14) Since the pulmonary circulation is under nervous and humoral control (3) and true pulmonary ischaemia in IRDS in all probability is mainly secondary to functional constriction it would seem natural that post mortem injection studies would fail to reveal the structural background of an underperfusion To study such a mechanism *in vivo* experiments seem desirable Recent attempts at micro angiography on living normal piglets have been successful (7)

The controversy concerning the structural background of the large right to left shunt known to be present in IRDS (19 23 27) should be settled for the time being The absence of arterio venous or large artery artery anastomoses in the human lung in IRDS as shown by micro angiography is noteworthy and the only explanation at present of a R L shunt *in the lung* is a passage of blood through non or poorly ventilated parenchyma The observations of arterio venous communications in the lungs by *Gronowski* (6) remain to be confirmed *Sobin et al* (21) using a silicone elastomer microvascular casting method did not find such anastomoses

It has been demonstrated here that there is no micro angiographical evidence of differences in pulmonary vasculature after pulmonary artery or pulmonary vein injection of lungs from infants with IRDS newborn infants without pulmonary lesions or stillborn infants This method of study is a valuable tool in many vascular contexts but for the vital question of the pathogenesis of IRDS functional and biochemical rather than morphological approaches to studies of the pulmonary vasculature and alveoli now appear to be more promising

SUMMARY

Nineteen pairs of lungs from perinatal autopsy cases were used for a standardized micro angiographic examination A contrast medium of 7 5 per cent barium sulphate in 6 per cent Rheomacrodex was injected with pulsatile flow for 30 minutes while the lungs were ventilated with negative pressure in a chamber After formalin fixation all lobes were micro angiographed and serially sectioned for microscopic examination The case series consisted of 5 pairs of lungs from stillborns 6 from newborns born alive and without pulmonary disease and 8 from infants dying from respiratory distress syndrome (IRDS) and presenting hyaline membranes The pulmonary arterial pattern was essentially similar in all groups No arteriovenous anastomoses were seen The filling of precapillary arterioles and capillaries was equally good in

ventilated and collapsed lung specimens There seems to be no vascular anatomical prerequisite in the neonatal lung for the development of IRDS

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EFFECTS ON THE ENDOCRINE PANCREAS IN CHINESE HAMSTERS FED ZINC DEFICIENT DIETS

By

LENNART BOQUIST and ÅKE LERNMARK

Received 19 xii 68

Zinc occurs in the endocrine pancreas of many species (*Maske* 1957 *Voigt* 1959) Following the discovery by *Scott* (1934) of the role of zinc for the crystallization of insulin the physiological relationship between zinc and insulin has been the subject of several investigations (*cf Vallee* 1959) It has been assumed that zinc is concerned with the occurrence or storage (*Maske* 1953 1957 *Logothetopoulos et al* 1964) and secretion (*Maske* 1953 1957) of insulin in the β cells The role of zinc in these processes remains however poorly understood (*Vallee* 1959 *Grodsky & Forsham* 1966) An association between zinc and glucagon in the islets has also been suggested (*cf Vallee* 1959) In diabetic patients an increased urinary excretion of zinc has been observed (*Tarui* 1963b) but it has not been possible to decide whether such hyperzincuria is the cause the consequence or an insignificant concomitant symptom of diabetes mellitus (*Constam et al* 1964) Increased concentration of zinc in the urine has also been found in alloxan diabetes (*Tarui* 1963a) The plasma zinc content in diabetics without complications has been reported to be increased (*Constam et al* 1964) or unchanged (*Vikbladh* 1951 *Prout et al* 1960 *Craig* 1962 *Davies et al* 1968) as compared with normal subjects *Todd et al* (1934) showed that zinc was essential in diets fed to rats Deficiency of zinc in this species has been observed to give impaired glucose tolerance (*Hove et al* 1937 *Quarterman et al* 1966) To the best of our knowledge there are no previous studies on the intravenous glucose tolerance of zinc deficient animals

The main purpose of the present work on the Chinese hamster was

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concentration before and rather soon after the administration of glucose the blood was sampled at 0 and 30 minutes of the experiments. The principles of the blood glucose determinations were the same as those previously described (*Boquist* 1967 c). For insulin assay the blood was collected in polyethylene micro tubes and allowed to coagulate at +8° C before centrifugation. Then the serum samples were frozen and stored at −25° C. The serum insulin level was determined with the double antibody radioimmunological technique (method C) of *Hales & Randle* (1963). Crystalline ox insulin (Lot No 819744 Vitrum AB Stockholm Sweden) with an activity of 24.8 U per mg was used as standard. The insulin antibodies and ¹²⁵I insulin were supplied by the Radiochemical Centre Amersham England. The assays were made in duplicates and the error of estimation was ± 5 per cent.

At predetermined intervals from 1 day to 4 months the animals were sacrificed and specimens were taken from the pancreas for light and electron microscopic examinations. The techniques used for morphological studies followed those of previous investigations (Boquist 1967 a and b). In addition a semi quantitative estimation of the pseudo isocyanin reaction (*Schiebler & Schiessler* 1959) was performed. In "unknown" random sections (5 μ thick) from the pancreas of 10 zinc deficient and 10 control I hamsters the metachromatic reaction in the β cells of 5 islets was classified as —, +, ++ or +++.

RESULTS

Tissue Concentration of Zinc

In Chinese hamsters fed the zinc deficient diet for 3 weeks the concentration of zinc in the testes was reduced as compared with the controls I (Table 1). No significant difference between the zinc deficient and control animals was found as to the concentration of zinc in heart, kidneys, liver and pancreas at that observation time.

TABLE 1

Zinc Concentration in Various Tissues of Chinese Hamsters Fed Zinc Deficient or Control Diet for 3 Weeks

Tissues	Zinc deficient diet	Control diet 1
Heart	33 ± 2 (7)	37 ± 4 (7)
Kidneys	45 ± 2 (8)	48 ± 3 (8)
Liver	116 ± 2 (10)	108 + 3 (7)
Pancreas	85 ± 3 (8)	87 ± 3 (6)
Testes	97 ± 6 (8)	14 ± 3 (7)

The levels have been calculated as μg/g wet weight and represent mean values ± S.E.M. The numbers of animals are given within brackets.
Control diet I refer to standard laboratory diet.

Urine Glucose

Glucosuria was not found in any of the zinc deficient or any of the 2 groups of control animals.

Blood Glucose Level

Hyperglycaemic animals were not observed. The fasting blood glucose values in the zinc deficient animals and in the 2 groups of control animals were all within the limits 102–121 mg/100 ml.

TABLE 2

Intraperitoneal Glucose Tolerance of Chinese Hamsters Fed Zinc Deficient or Control Diets for Three Weeks

Diets	Time after administration of glucose							
	Minutes			Hours				
	0	1	0	1	2	4	6	8
Zinc deficient diet	108 ± 1.4 (28)	183 ± 3.4 (9)	219 [illegible] (1)	234 ± [illegible] (11)	244 ± 6.7 (8)	165 ± 3.0 (10)	1[illegible] ± 3.4 (9)	115 ± 3.1 (19)
Control diet I	10[illegible] ± 2.[illegible] (10)	1[illegible]5 [illegible]	1[illegible] (9)	172 [illegible] 3.0	111 ± 3.7 (9)	104 ± [illegible] (8)	110 ± 2.[illegible] (6)	104 ± 3.3 (6)
Control diet II	106 ± 2.4 (10)	1[illegible]9 [illegible]	1[illegible] 3.3 (9)	118 ± 3.1 (6)	103 ± 1.8 (9)	10[illegible] ± 2.9 (6)	104 ± 3.2 (6)	105 ± 3.3 (8)

The blood glucose levels [illegible] mg 100 ml and represent mean values ± S.E.M.
The numbers of animals are given within brackets.
Control diet I refers to standard laboratory diet.
Control diet II refers to zinc deficient diet with the addition of zinc in water

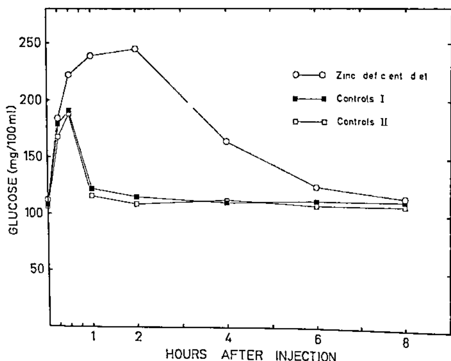


Fig 1

The intraperitoneal glucose tolerance of 3 groups of Chinese hamsters fed experi mental diets for 3 weeks is shown Glucose at a concentration of ? g/kg of body weight was injected intraperitoneally into 28 hamsters fed a zinc deficient diet 10 hamsters fed a standard laboratory diet (controls I) and 10 hamsters fed the zinc def cient diet with the addition of zinc (controls II) Blood glucose determinations were performed on a varying number of animals at 0 15 and 30 minutes as well as at 1 ? 4 6 and 8 hours The heavy black lines denote the mean blood glucose levels for the 3 animal groups

Intraperitoneal Glucose Tolerance

In the hamsters fed the zinc deficient diet decreased glucose toler ance was observed already after this diet had been fed for one week Repeated tests at various time intervals showed persistent pathological values Though rather wide individual variations occurred no obvious tendency to a higher or lower degree of decreased glucose tolerance was present at repeated tests There was no difference between males and females nor were there any differences between younger and older animals The glucose tolerance curves for the controls I and II were rather similar and showed a peak value of about 180 mg/100 ml at 30 minutes and then a rather rapid return to the normal level In Table 2 and Fig 1 the results of the glucose tolerance tests on hamsters fed the experimental diets for 3 weeks are given

Intravenous Glucose Tolerance

Already when the diets had been fed for 1 and 2 days the blood glucose level at 30 minutes after the administration of glucose was increased in the zinc deficient animals as compared with the controls I. The values at 0 minute showed no difference between the animal groups. At 7, 11, 21, 3[illegible] and 58 days there was no difference between the blood glucose level of the animal groups at 0 minute whereas the level at 30 minutes was increased in the zinc deficient hamsters. It thus seemed that the intravenous glucose tolerance was decreased in the zinc deficient hamsters. As in the intraperitoneal tests no sex or age differences but rather wide individual variations of the blood glucose values were found. The results have been summarized in Table 3.

TABLE 3

Intravenous Glucose Tolerance [illegible] Hamsters Fed Zinc Deficient [illegible] Diets

Diets	Time of feeding the experimental diets					
	1 [illegible] days		[illegible] weeks		5 8 weeks	
	Minutes		Minutes		Minutes	
	0	[illegible]	0	30	0	30
Zinc deficient diet	10[illegible] [illegible] 2 9	72 [illegible]	10[illegible] ± [illegible]	[illegible] ± [illegible]	107 ± 2.0	2[illegible] ± 5 1
	(13)		([illegible])		([illegible])	
Control diet 1	10[illegible] ± 3 4	1[illegible]	[illegible]	[illegible]	107 ± 3 7	133 ± 3 9
	(7)		([illegible])		(4)	

The blood glucose levels at 0 and 30 minutes have been calculated as mg 100 ml and represent the mean values ± S.E.M. The numbers of the animals are given within brackets. Control diet I refers to standard laboratory diet.

TABLE 4

Serum Immunoreactive Insulin [illegible] before and after (30 Minutes) Intravenous Glucose (minute [illegible]) Hamsters Fed Zinc Deficient [illegible] Diets

Diets	Time of feeding the experimental diet					
	1 7 days		3 [illegible] weeks		5 8 weeks	
	Minutes		Minutes		Minutes	
	0	30	0	30	0	30
Zinc deficient diet	3 1 ± 0 7	[illegible]1 ± 0 [illegible]	1 [illegible] ± 0 6	1 9 ± 0 [illegible]	3[illegible] ± 1 3	4 9 ± 1 7
	(13)		([illegible])		([illegible])	
Control diet I	1 4 ± 0 5	2 5 ± 0 7	0 [illegible] ± 0 [illegible]	[illegible]0 ± 0 2	2 6 ± 0 7	1.5 ± 0 7
	(7)		(7) p < 0 05		(4)	

The concentrations have been calculated as [illegible] insulin/ml serum measured as ox insulin and represent the mean value ± S.E.M. Where the difference between the value at 0 and 30 minutes within one time and dietary group is statistically significant the p value is included. The numbers of the animals are given within brackets. Control diet I refers to standard laboratory diet.

Serum Immunoreactive Insulin

The results of the serum insulin determinations before and 30 minutes after the intravenous administration of glucose have been summarized in Table 4 In the zinc deficient hamsters there was no statistically significant difference between the values at 0 and 30 minutes in any of the 3 time groups The insulin value at 30 minutes was higher than at 0 minute in the hamsters fed the control diet I for 2–3 weeks In the two other time groups of the controls I there was no statistically significant difference between the values at 0 and 30 minutes As to the serum insulin concentration before the administration of glucose there was no statistically significant difference between the animal groups

Light Microscopic Findings

The general appearance of the islets was normal No infiltration of inflammatory cells was present Decreased granulation of the β cells occurred rather often in the zinc deficient animals The sulphide silver method for light microscopic study of heavy metals was positive in the central and peripheral region of the islets both in zinc deficient and control hamsters Though the positive reaction of this method some

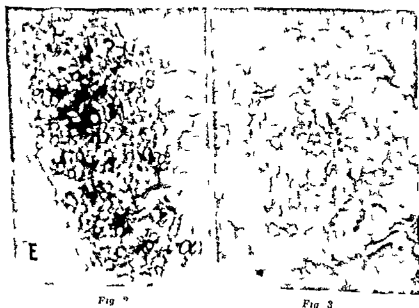


Fig 2 Pancreatic islet of a Chinese hamster fed control diet I for 2 weeks showing metachromatic reaction with pseudoisocyanin in the central β-cells (β) The peripheral α-cells (α) and the exocrine parenchyma (E) are unaffected Bouin's fixative Pseudo isocyanin procedure × 200

Fig 3 Pancreatic islet of a Chinese hamster fed zinc deficient diet for 2 weeks showing almost no metachromatic reaction with pseudo isocyanin Bouin's fixative Pseudo isocyanin procedure × 300

Intravenous Glucose Tolerance

Already when the diets had been fed for 1 and 2 days the blood glucose level at 30 minutes after the administration of glucose was increased in the zinc deficient animals as compared with the controls I. The values at 0 minute showed no difference between the animal groups. At 7, 11, 21, [illegible] and 58 days there was no difference between the blood glucose level of the animal groups at 0 minute whereas the level at 30 minutes was increased in the zinc deficient hamsters. It thus seemed that the intravenous glucose tolerance was decreased in the zinc deficient hamsters. As in the intraperitoneal tests no sex or age differences but rather wide individual variations of the blood glucose values were found. The results have been summarized in Table 3.

TABLE 3

Intravenous Glucose Tolerance of Chinese Hamsters Fed Zinc Deficient or Control Diets

Diets	Time of feeding the experimental diets: 1–7 days, Minutes		2–3 weeks, Minutes		5–8 weeks, Minutes	
	0	30	0	30	0	30
Zinc deficient diet (13) / (9) / (7)	105 ± 2 9	227 ± 4 2	106 ± 2 4	198 ± 5 7	107 ± 2 0	221 ± 5 1
Control diet I (7) / (7) / (4)	106 ± 1 4	180 ± 2 1	109 ± 2 1	177 ± 2 8	107 ± 3 7	183 ± 3 9

The blood glucose levels at 0 and 30 minutes have been calculated as mg/100 ml and represent the mean values ± S.E.M. The numbers of the animals are given within brackets. Control diet I refers to standard laboratory diet.

TABLE 4

Serum Immunoreactive Insulin Level before (0 Minute) and after (30 Minutes) Intravenous Glucose Administration in Chinese Hamsters Fed Zinc Deficient or Control Diet

Diets	Time of feeding the experimental diets: 1–7 days, Minutes		2–3 weeks, Minutes		5–8 weeks, Minutes	
	0	30	0	30	0	30
Zinc deficient diet (13) / (9) / (7)	3 1 ± 0 7	2 1 ± 0 6	1 8 ± 0 6	1 9 ± 0 9	3 2 ± 1 3	4 9 ± 1 7
Control diet I (7) / (7) p < 0 05 / (4)	1 4 ± 0	2 5 ± 0 7	0 9 ± 0 2	2 0 ± 0 2	2 6 ± 0 7	1 5 ± 0 7

The concentrations have been calculated as ng insulin/ml serum measured as ox insulin and represent the mean values ± S.E.M. Where the difference between the value at 0 and 30 minutes within one time and dietary group is statistically significant the p value is included. The numbers of the animals are given within brackets. Control diet I refers to standard laboratory diet.

times was weaker in the zinc deficient animals this did not allow any estimation of the zinc content in the islets not even semi quantitatively No degenerative changes of the β cells were recorded The α_1- and α cells were unaffected

The pseudo isocyanin procedure for insulin was often negative in the zinc deficient animals as compared with the controls (Figs 2 and 3) The semi quantitative estimation of the results of the pseudo isocyanin procedure gave the following results

	—	+	++	+++
Zinc deficient hamsters	3	4	2	1
Control I hamsters	0	2	2	6

Electron Microscopic Findings

The β cells of the zinc deficient animals often showed well developed endoplasmic reticulum and Golgi complex as well as degranulation (Fig 4) The other organelles were unaffected in these cells Th α_1 α and agranular cells were unchanged in the zinc deficient animals In the control hamsters no ultrastructural alterations were recorded

DISCUSSION

In rats fed the same zinc deficient diet and given the same water as that used in the present study clear signs of zinc deficiency appeared after 3 weeks (*Bergman et al* 1968) The Chinese hamsters fed the zinc deficient diet showed decreased zinc concentration in testes but not in heart liver kidneys and pancreas This conforms to findings in zinc deficient rats where the concentration of zinc is markedly reduced in testes and bones but not in other tissues (*Macapinlac et al* 1966) Thus it seems that the zinc deficient diet used in the present work was effective This is of value to know as it is known that a great risk of contamination by zinc is involved in biological experiments (*Anonymous* 1968)

In the urine and blood glucose determinations as well as in the intraperitoneal glucose tolerance tests where two kinds of control diets were used any difference between the effects of these diets was not found Because of this the use of only one kind of control diet for the atomic absorption spectrophotometry the intravenous glucose tolerance tests and the serum insulin determinations seems to be justified

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Fig 4

Portion of pancreatic islet of a Chinese hamster fed zinc deficient diet for 1 week demonstrating two α_2-cells (α) and one agranular cell (γ) without alterations In most of the β-cells (β) the occurrence of secretory granules is rather sparse In one of the β-cells there is a cilium (Ci) protruding into an intercellular space × 4 000

Zinc deficient rats exhibit anorexia and reduced feed efficiency (*Prasad et al* 1967). It has also been found that the glucose tolerance curve of control rats fed restricted diets is low and flat as compared with that of zinc deficient rats (*Macapinlac et al* 1966). Since pathological changes including elevated glucose tolerance curves appeared in Chinese hamsters fed zinc deficient diet but not in those fed standard laboratory diets or zinc deficient diets with the addition of zinc it seems to be more probable that the zinc deficiency *per se* is the cause of these effects and that possible differences in dietary intake play a subordinate role. The fact that no age or sex differences were found in the present study seems also to support the view that deficiency of zinc was responsible for the pathological alterations and it indicates that no age or sex factors interfered with the blood glucose as in rats (*Hajdu & Rona* 1967, *Kumar* 1968). As to the plasma zinc concentration in man it is known that sex and age differences are insignificant (*Davies et al* 1968).

Since there were neither hyperglycaemia or glucosuria nor any marked morphological islet lesions in the Chinese hamsters fed the zinc deficient diet, it is obvious that the frequency of spontaneously occurring overt diabetes mellitus in this species could not be increased by feeding such a diet, at least not with the present observation times. On the other hand the occurrence of decreased glucose tolerance in these hamsters might indicate that zinc deficiency evoked a prediabetic state. Similarly decreased glucose tolerance was found in the Chinese hamster also after pancreatectomy (*Boquist* 1967c) and after the administration of alloxan (*Boquist* 1968).

Hove et al (1937) observed that the oral glucose tolerance curves were irregular and delayed in zinc deficient rats, whereas the glucose level and liver glycogen were normal. This was interpreted as indicative of impaired absorption of glucose from the bowel. In the Chinese hamsters fed the zinc deficient diet both the intraperitoneal and intravenous glucose tolerance was impaired. Thus it seems that the absorption of glucose from the peritoneal cavity is not conspicuously altered in these animals. As a consequence of this the intraperitoneal glucose tolerance tests were considered to be representative.

On the basis of a study of 4 pairs of zinc deficient and control rats *Quarterman et al* (1966) have stated that the zinc deficient animals had decreased concentration of plasma insulin. These authors also suggested that the rate of secretion of insulin in zinc deficient rats was decreased in response to glucose stimulus. In the present study there was no significant difference in the serum insulin levels in zinc deficient and control animals and the values conform to those found by *Gerritsen & Dulin* (1967) in nonketotic non diabetic Chinese hamsters. The interpretation of the responses of the serum insulin to intravenous glucose administration is somewhat difficult. No difference between the values at 0 and 30 minutes was observed in the zinc deficient hamsters

seemingly indicating that there is no such response in these animals. In the control hamsters, on the other hand, the values at 0 and 30 minutes were found to differ in one of the three time groups, where no difference occurred in the other two groups. The cause of these equivocal findings is still unknown and will be further analysed in a forthcoming work.

The plasma zinc concentration in man has been said to show only small variations normally (*Davies et al.* 1968), possibly because of an efficient homeostatic mechanism for zinc (*Anonymous* 1968). Oral or intravenous glucose load in man induces a rapid fall of the plasma zinc level followed by a rather rapid recovery (*Davies et al.* 1968). In pregnant female rats and in growing male rats *Dreosti et al.* (1968) observed a fall of the plasma zinc concentration already after a zinc deficient diet had been fed for one day. After that time the plasma zinc decrease became progressively less marked and a plateau was reached by about 7 days. This was suggested to be due to the inability of these rats to mobilize their body deposits adequately to accomodate for even a short period of dietary zinc restriction. The glucose tolerance tests in the present study show that also the Chinese hamster may react rapidly to dietary zinc deficiency and that there are no marked alterations in short or long time experiments. The cause of the impaired glucose tolerance of the zinc deficient Chinese hamsters is not known. Though there was no decrease of the serum insulin concentration in these animals the light and electron microscopic examinations indicate that the β-cells had a decreased granulation and thus possibly also a decreased amount of insulin. This possibility is supported by the results of the semi quantitative estimation of the pseudo isocyanin procedure. Though the light microscopic sulphide silver reaction did not allow any quantitative metal estimation and though the atomic absorption spectrophotometry did not reveal any decreased concentration of zinc in the pancreas, it seems, however, possible that there might be a decreased content of zinc in the islets of the zinc deficient animals. The ultrastructural finding by *Pihl & Falkmer* (1967) of a decreased amount of sulphide silver positive secretion granules in the β cells of zinc deficient rats may give some support to this view. If the concentration of zinc in the islets is diminished and if it is accepted that zinc is concerned with the storage of insulin in the β cells, it may be speculated that the amount of stored insulin is decreased in zinc deficient animals. When excess glucose is administered to these animals, as in glucose tolerance tests, an insufficient amount of insulin may be available resulting in pathological effects on the glucose tolerance curves. When glucose is not given in excess to the animals, the production and secretion of insulin seem to be sufficient and no hyperglycaemia or glucosuria is observed. The lack of insulin response to glucose in the zinc deficient animals and the occurrence of this response in some of the control hamsters might support these speculations. As it is known that a de-

creased tissue concentration of zinc in zinc deficient rats is combined with decreased activities of certain enzymes (*Prasad et al* 1967) the effect of zinc deficiency in the Chinese hamster may also be thought to be due to enzymatic derangement Further studies are required to test the validity of these speculations

SUMMARY

Three groups of Chinese hamsters were fed a zinc deficient diet a standard laboratory diet and a zinc deficient diet with the addition of zinc in water respectively By atomic absorption spectrophotometry the concentration of zinc was determined in samples from heart kidneys liver pancreas and testes of animals fed the experimental diets for 3 weeks A decreased concentration of zinc was found in the testes indicating that there actually was an effect of the zinc deficient diet

Glucosuria or hyperglycaemia could not be elicited by feeding the zinc deficient diet The intraperitoneal and intravenous glucose tolerance was impaired in hamsters fed the zinc deficient diet The serum immunoreactive insulin level in zinc deficient hamsters did not deviate from that in the control animals In hamsters fed the zinc deficient diet no significant increase of this level occurred after glucose injection

In the light and electron microscopes decreased granulation of the islet β cells of zinc deficient hamsters was observed The pseudo isocyanin method for insulin disclosed weaker reaction in the β-cells of animals fed the zinc deficient diet The α_1 α and agranular cells showed no morphological alterations

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GRANULOMATOUS HYPOPHYSITIS AND THYROIDITIS WITH LYMPHOCYTIC ADRENALITIS

By

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In rare cases the pituitary gland of elderly women may show characteristic granulomas not only with lymphocytic infiltration and epithelioid cells but also with true giant cells Independently of the granulomas giant cells are present also in intact glandular tissue The granulomas are reminiscent partly of miliary tubercles but are entirely unrelated to tuberculosis or to syphilis (Simmonds 1917) *Sheehan & Summers* in 1949 collected 18 such cases including Simmonds 4 as pituitary giant cell granulomas They concluded that the group may include lesions due to a number of different causes such as syphilis tuberculosis or sarcoidosis but that in the majority of cases they appear to represent a specific disease process whose aetiology is unknown

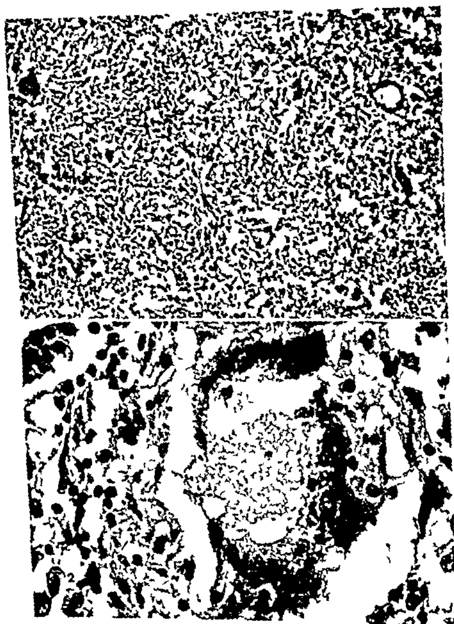
It is our impression from the recent literature that the granulomatous pituitary lesions with giant cells may be divided into 3 groups

(1) Granulomas due to tuberculosis syphilis or sarcoidosis
(2) Granulomas of unknown aetiology with secondary changes in other organs especially the thyroid and adrenals as a consequence of pituitary insufficiency—or without such secondary lesions if only a minor part of the hypophysis is involved
(3) Granulomas of unknown aetiology combined with changes in other organs especially the thyroid and adrenals which can hardly be secondary to pituitary insufficiency mainly because the histological appearances in these organs are of a type entirely different from that of changes caused by impaired pituitary function

The object of the present paper is to report and discuss a case of the last mentioned group

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Figs 1 2

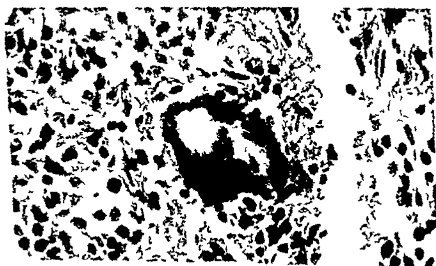
Fig 1 Pituitary gland Ill defined confluent epithelioid cell granulomas Giant cells of varying size and appearance Lymphocytic infiltration × 125 Haematoxylin eosin

Fig 2 Pituitary gland Vacuolized giant cell with marginal irregularly distributed nuclei of varying size × 500 Haematoxylin eosin



Fig 4

Thyroid gland Granulomatous area Vacuolized giant cell Lymphocytic infiltration × 1[illegible] Haematoxylin-eosin



Fig

Thyroid gland Giant cell with irregularly distributed partly marginal nuclei of varying size × 500 Haematoxylin eosin

was preserved the follicles were on the whole small with sparse colloid and more or less enlarged epithelial cells with eosinophilic cytoplasm Here and there granulomas corresponding to those found in the pituitary both in respect to giant cells and epithelioid cell granulomas (Figs 4 and 5)

Case Summary Autopsy on a 74 year old woman who had exhibited signs of pluriglandular insufficiency revealed in the pituitary giant cell granulomas in the thyroid goitre showing partly granulomas of the same type and partly changes as in Hashimoto's thyroiditis while the adrenals exhibited lymphocytic infiltration Histological examination of the lymph nodes skin subcutaneous tissue brain tissue leptomeninges myocardium liver spleen and kidneys showed no abnormalities

DISCUSSION

There is no evidence to indicate that the pathological changes of the pituitary gland were caused by tuberculosis sarcoidosis or syphilis Owing to the localization the named diseases would be expected to have been generalized In addition sarcoidosis involving the pituitary will in more than 90 per cent give rise to diabetes insipidus with involvement of the posterior lobe syphilis usually affects the entire pituitary and tuberculosis seldom causes hypopituitarism (16) Furthermore the morphological similarity to the three diseases is fairly modest In our case the giant cells were of extremely varying size and nuclearity partly with irregularly distributed nuclei and in places with coarsely vacuolized cytoplasm The epithelioid cells were strikingly plump and the granulomas ill defined We found no hyalinosis fibrosis caseous necroses or gumma formation The histological appearances corresponded accurately to previously described cases of pituitary giant cell granulomas of unknown aetiology

Sheehan & Summers (1949) reported two cases of chronic fibrous lesions in the pituitary gland which they interpreted as sequelae to cranial injury in particular with fracture of the base of the skull These authors suggested that there might be a question of healed granulomas Our patient had a history of cranial injury 10 years before her death but it seems unlikely that this trauma can be related to the active granulomatous process which was entirely devoid of fibrosis

It is not likely either that the appreciable changes in the adrenals could have been secondary to pituitary insufficiency True the patient had reduced function of the anterior pituitary lobe This is indicated by the clinical findings as well as by the fact that more than three quarters of the anterior lobe had undergone destruction (21) But apart from doubtful narrowing of the adrenal cortex there was well marked lymphocytic infiltration which does not accord with the usual adrenal reaction to hypopituitarism On the basis of the clinical signs as well as the histological appearances it must be considered likely that the entire disease ran a fairly brief course so that the usual secondary adrenal changes did not have time to develop It is more difficult to appraise the thyroid changes in relation to pituitary giant cell granulomas but they differed in essential respects from the atrophy fibrosis

and lymphocytic infiltration often seen secondary to reduced pituitary function In our case there was mild goitre which presented itself mainly as Hashimoto s thyroiditis but here and there the thyroid showed changes identical with those found in the pituitary and entirely unlike de Quervain s granulomatous thyroiditis

It is worth considering whether this case may have represented an extended Schmidt syndrome involving the pituitary *Schmidt*, in 1926 described two patients with hypofunction of the adrenals as well as of the pituitary gland The adrenals showed lymphocytic infiltration and atrophy as in idiopathic Addison s disease and the thyroid changes reminiscent of Hashimoto s thyroiditis Later *Bloodworth et al* (1954) found signs of hypothyroidism in 13 out of 35 patients with primary adrenal insufficiency *Kracht & Hachmeister* (1966) interprete Schmidt s syndrome as the link between auto immune thyroiditis and those types of primary adrenocortical atrophy which have been considered an auto immune disease *Nerup et al* (1966) found antibody to cytoplasmic antigen in the adrenal cortex of 31 out of 48 patients with "idiopathic Addison s disease Of these 31 patients 21 also had thyroid antibodies *Bl ard et al* (1967) demonstrated the same antibody combination in 12 out of 64 patients with adrenal insufficiency They pointed out that among 57 patients with Hashimoto s disease none had adrenal antibodies and that the incidence of Addison s disease in patients with primary myxoedema is low Therefore the autoimmune processes which affect the two glands appear to represent a distinct disease entity and this is further supported by the fact that the antibodies are organ specific We have no proof that our case might be a triglandular Schmidt s syndrome with auto immune processes directed against all three glands but below we shall review certain morphological studies which have given rise to reflections in this direction

Goudie & Pinkerton (1962) suggested the possibility of auto immunization in a case of hypophysitis and thyroiditis The thyroid changes were reminiscent of ours consisting in Hashimoto s thyroiditis with giant cells but the hypophysis showed severe lymphocytic infiltration without giant cells The adrenals could not be found *Hume & Roberts* (1967) have adduced similar suggestions on the basis of a case of lymphocytic hypophysitis and thyroiditis (without giant cells) combined with adrenal atrophy and pernicious anaemia *Oelbaum & Wainwright* (1950) reported a case of typical giant cell granulomas associated with multiple granulomas of the same type in the adrenals which were moreover atrophic The thyroid exhibited atrophy fibrosis and lymphocytic infiltration These authors did not further discuss the aetiology but stated that the degree of atrophy in the secondary endocrine organs was not of the advanced type seen following long lasting hypopituitarism This was in keeping with the clinical biochemical and histological studies which indicated that the pituitary lesion was subacute About the same applies to our case only more so

Donnach & Wright (1951) in one of their two cases of pituitary giant cell granulomas found granulomas of exactly the same histological architecture in the adrenals which also showed atrophy. The thyroid exhibited only adenomata. These authors too did not enter into any aetiological details. *Morgenstern* (1961) considered the possibility of autoimmunization in a patient with pituitary giant cell granulomas combined with granulomatous thyroiditis. He also found numerous granulomas in the myocardium and a few in the kidneys and adrenals. *Hirsch & Robbins* (1952) published 4 cases of giant cell granulomas. Three were presumably due to sarcoidosis but this could be ruled out in the fourth case. The adrenals were atrophic with severe diffuse lymphocytic infiltration. The thyroid gland was not studied. *Subbuswamy et al* (1967) reported one case of pituitary giant cell granulomas in which the thyroid showed granulomas of the same type. The adrenals were grossly normal but nothing is stated concerning histological findings. Since the pituitary changes were slight and involved only a small part of the gland these authors did not interpret the thyroid lesions as being secondary to the pituitary giant cell granulomas, feeling that the pathological appearances were more likely to be due to a common agent of unknown nature.

All the reported cases of pituitary giant cell granulomas have had only deficient or no serological studies with a view to autoimmunization. In our case the antinuclear factor, antihuman globulin consumption test, and study for cytoplasmic thyroid antibody by immune fluorescence technique were negative. The last mentioned test is positive in about 90 per cent of sera from patients with diffuse chronic thyroiditis of the Hashimoto type and in about two thirds of sera from patients having acquired myxoedema without goitre (*Halberg* 1967). This would seem to indicate that autoimmune thyroiditis was rather unlikely in our patient. Moreover *Goudie* (1968) and *Nerup et al* (11) did not find specific pituitary antibodies in patients with idiopathic panhypopituitarism but it is not known whether these materials included cases of pituitary giant cell granulomas. There is the possibility that the present technique is not able to demonstrate antibodies in pituitary giant cell granulomas. Goudie (1962, 1968) feels that the purely lymphocytic type of hypophysitis is presumably of autoimmune nature and this assumption is supported by experimental investigations (11) while pituitary giant cell granuloma is perhaps an entirely different disease and that at present it is not possible to decide its nature. He believes there is a possibility that pituitary giant cell granuloma is an autoimmune disease in which the morphological signs of the immune reaction differ considerably from that found in most other autoimmune diseases. He goes on: *I should however say that granulomata are found in the liver in primary biliary cirrhosis which certainly is associated with non organ specific autoantibody formation and I recently encountered at necropsy a*

case of idiopathic adrenal atrophy in which strong adrenal auto antibody was definitely present and in which a single sarcoid like follicle was present in the small amount of adrenal cortex which survived (7) Our case had granulomatous hypophysitis and thyroiditis but also thyroid and adrenal changes of the type seen in auto immunization The thyroid and adrenal changes cannot be interpreted as a consequence of pituitary insufficiency but all three lesions are presumably concurrent

SUMMARY

A case of hypophysitis thyroiditis and adrenalitis in a patient with signs of insufficiency of the anterior pituitary lobe is reported The pituitary changes were typical of the granulomatous hypophysitis with giant cells The thyroid gland exhibited the same histological appearances and besides lesions like those seen in Hashimoto s thyroiditis In the adrenals lymphocytic infiltration was present It is considered likely that the disease has been rather short lasting and that the named organic changes are secondary to the same action which is unknown but the possibility of auto immunization is ventilated

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ACTINOMYCES IN TONSILLAR TISSUE

A Histological Study of a Tonsillectomy Material

By

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Received 8 xi 68

Actinomyces as the cause of human disease has been known for 90 years Its habitat is the oral cavity It is frequently found in carious teeth dental calculus (tartar) and occasionally in the tonsils

Actinomycosis may affect any organ or tissue More than 50 per cent of the cases however are located to the face and neck Among out patients the cervicofacial form is even more predominant

Actinomyces is considered as an opportunistic microbe (*McKinnon* 1962 *Peabody & Seabury* 1960 *Ulx* 1962) *Israel* (1885) held the oral cavity to be the chief reservoir *Ruge* (1885) found the granules in tonsillar crypts but did not believe them to be true *Actinomyces* colonies *Lord* (1910) and *Emmons* (1938) isolated *Actinomyces* from tonsillar tissue *Emmons* found the hyphae by direct examination in 74 of 200 pairs of tonsils (37 per cent) and by culture in 14 per cent in a New York material *Wilkinson* (1929) examined the tonsils of 10 000 cases of tonsillectomy performed at the Mayo Clinic 1923–26 By histological examination he found granules resembling *Actinomyces* in 177 cases (1 8 per cent)

The frequencies observed by *Wilkinson* and by *Emmons* differ considerably Information on *Actinomyces* granules according to age and sex is scarce Further study therefore seemed warranted

MATERIAL AND METHODS

During 1967 tonsils from 103 patients were received in the Department of Pathology Ullevål Hospital The material came from the Department of Oto Rhino Laryngology In two cases only one tonsil was extirpated

The indications for removal are given in Table 1

For routine diagnosis the tonsils were fixed in 4 per cent neutral formaldehyde solution for about 24 hours Each tonsil was transsected and a central slice embedded in paraffin Two sections about five microns thick were stained with haematoxylin and eosin (H E)

The sections have been re-examined for *Actinomyces* colonies In a number of cases the preliminary diagnosis was doubtful In these cases and in all cases were a tentative positive diagnosis had been given new sections were cut and stained with Gram s stain and with periodic acid *Schiff* (PAS)



Fig 1

Actinomyces colonies in tonsillar tissue H E stain Magnification × 75



Fig 2

The Ray fungus with the *hyphae* arranged as spokes in a wheel Gram's stain Magnification × 5[illegible]0

TABLE 1

Indications for Tonsillectomy Number of Cases

Chronic tonsillitis	86
Chronic tonsillitis and adenoids	12
Glomerulonephritis	2
Peritonsillar abscess	2
Diagnostic in brain tumour (craniopharyngeoma)	1
Total	103

Identification

The typical picture of an Actinomyces colony is that of the ray fungus. The branching bacterial bodies—*hyphae*—are intermingled and arranged like spokes in a wheel (Figs. 1 and 2). In good sections with no shrinkage the clublike ends of the *hyphae* can be seen in the periphery of the colonies.

Actinomyces is a Gram positive organism. Central portions of large colonies are sometimes Gram negative. In H.E. sections the colonies are dark with a slight bluish tint. Like most microfungi Actinomyces takes the PAS stain which makes the colonies stand out conspicuously so that they are easily recognized.

RESULTS

In all cases cellular debris and bacterial colonies were found in the crypts. *Actinomyces* colonies were found in 17 of the 103 cases. This is a minimum number as serial sectioning has not been carried out. The age and sex distribution is given in Table 2. Uncertain colonies (numbers in brackets) either had a doubtful *Gram* reaction or seemed to be broken up by a crushing trauma during extirpation. Some small colonies did not show the typical picture of spokes in a wheel.

TABLE 2

Actinomyces *Colonies in 103 Consecutive Tonsillectomy Cases According to Age and Sex. Doubtful Colonies within Brackets*

Age interval in years	Males Cases	Males Colonies	Females Cases	Females Colonies	Total Cases	Total Colonies
Under 5	8	1	4	0	12	1
5–9	9	1	5	0	14	1
10–14	9	1	8	1 (1)	17	2 (1)
15–19	10	1 (1)	26	7 (2)	36	8 (3)
20–24	6	1	13	3 (1)	19	4 (1
25–37	4	0 (1)	1	1	5	1 (1)
Total	46	5 (2)	57	12 (4)	103	17 (6)

An increasing number of colonies are found after the age of 15 (Table 3). This increase does not quite attain significance at the conventional level of 5 per cent probability.

In 101 of the cases the histological diagnosis was chronic tonsillitis /

hyperplasia of tonsillar tissue In one case the tonsillitis was subacute and in another an abscess wall was found

The *Actinomyces* granules are easily overlooked by routine examination In none of the 17 cases were the colonies recognized at the first examination In 6 of them however fungus colonies were described

TABLE 3

Actinomyces *Colonies before and after 15 Years*

Age group	Colonies	No colonies	Total
14 – under	4 (9.3%)	39	43
15 – over	13 (21.7%)	47	60
Total	17 (16.5%)	86	103

P = 0.08 (Fisher Irving test)

DISCUSSION

Actinomyces very seldom affects the lymphatic system (*Pope* 1938) It is customary to regard the *Actinomyces* granules found in tonsils as mere saprophytes It should be obvious however that neither the present material nor any other tonsillectomy material reported in the literature can be considered normal The possibility that the granules may be connected with chronic tonsillitis cannot be excluded without consideration

In *Williamson*'s material collected in the Mid Twenties the age and sex *distribution is not given in detail* Although his method of examination hardly differs from the present it is therefore somewhat doubtful to compare it with the present material The difference in frequencies however would statistically be found highly significant ($\chi^2 = 11.5 > \chi^2_{0.001} = 10.827$)

Emmons (1938) cut the tonsils in thin slices and examined the contents of the crypts by direct examination In this way he found *Actinomyces* in 37 per cent of the cases He admits the possibility however that some of the filamentous organism seen and listed as *Actinomyces* were not correctly identified and thinks this may partly explain why he only found *Actinomyces* by culture in 17 per cent of the cases His material can because of the difference in methods and aim hardly be compared with the present material

Williamson (1929) on the other hand looked for granules and the difference between his and the present material requires an explanation

1 Either *Williamson* set up far more strict criteria for what he considered to be colonies According to his illustrations this is hardly the case 2 Or the proportion of children was higher Although the number of children in which granules were found is not stated the distribution in the total material is much the same 3 Due consideration should

also be given to the possibility that he may just have overlooked the colonies but 4. The fact that indications for tonsillectomy are much stricter now than four decades ago may offer the explanation. If fewer normal tonsils are removed now, the higher percentage of *Actinomyces* colonies may indicate that they are a sign of disease in the tonsils.

Histological examination shows on the other hand no distinct difference between tonsils containing granules and the rest of the material. Fibrosis, one of the hallmarks of actinomycosis, is not conspicuous. There is therefore not sufficient reason to suppose that chronic tonsillitis is actinomycotic. The possibility should be considered, however, that the granules may act as foreign bodies like *Soderlund* (1927) claimed they could do in salivary ducts. His theory that salivary duct calculus are calcified *Actinomyces* colonies has on the other hand not been confirmed by *Husted* (1953).

Tonsils as the port of entry in actinomycosis has been described and in a few cases the disease started after tonsillectomy (*Cope* 1938, *Harvey et al.* 1959, *Poncet* 1904).

The age difference in the present material compares with the distribution found in actinomycosis. The majority of cases occur between the age of 15 and 35. Less than 3 per cent occur in children under the age of ten (*Cope* 1938).

The sex proportion found, 5 males and 12 females, does not conform with the distribution in actinomycosis, which is "something like two males to one female" (*Cope* 1938).

SUMMARY AND CONCLUSION

Tonsils from 103 tonsillectomy cases were re-examined histologically for *Actinomyces* granules. A positive identification was made in 17 cases. PAS staining was found excellent for screening purposes.

The infestation rate was higher after puberty than before and showed an age distribution similar to that in clinical actinomycosis. The sex proportion did not show the male predominance characteristic of the disease.

Neither the present nor any other tonsillectomy material is "normal". The possibility cannot be excluded that *Actinomyces* colonies may be associated with tonsillar disease, either as a cause or as a consequence.

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THE SPECIFICITY OF CIRCULATING ANTIBODIES IN EXPERIMENTAL INFECTIONS WITH *MYCOBACTERIUM BOVIS* AND *MYCOBACTERIUM TUBERCULOSIS* DEMONSTRATED BY IMMUNOFLUORESCENCE

By

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In a previous paper (1) the presence of circulating antibodies in rabbits infected with *M avium* and *M bovis* was demonstrated by means of immunofluorescence The antibodies produced during infection with *M avium* possessed a high degree of type specificity which finding led to the elaboration of a method for serological type identification of avian and avian like mycobacteria (2)

The present study concerns investigation into the specificity of antibodies in rabbits infected with *M bovis* (virulent and attenuated (BCG)) and *M tuberculosis* The three parts of which it is composed are

A A methodological study of the fluorescence antibody test (FAT) concluding with a detailed description of the technique used subsequently

B Immunization schedules serum production and specificity of antisera

C Serotyping wild strains of *M bovis* and *M tuberculosis* isolated from Danish patients

PART A

METHODOLOGICAL STUDY OF THE FLUORESCENCE ANTIBODY TEST (FAT)

The technique described in previous papers (1 2) was based mainly on studies with *M avium* and was found inadequate for the investigation of mammalian mycobacteria A positive FAT is read as a yellowish green emission from the bacilli when the smear is excitated with a bluish ultraviolet beam When the FAT is negative *M avium* and most atypical mycobacteria emit a bluish autofluorescence which is easily

distinguishable from the positive reaction while mammalian mycobacteria emit a greyish white autofluorescence which makes differentiation difficult. Furthermore, it was found difficult to keep the bacilli on the slides during the different stages of the staining procedure. This phenomenon was particularly evident in the positive reactions, thus resulting in a number of false negatives.

Various conditions for the FAT were therefore investigated, basing the evaluation of their suitability on the brightness of the bacilli stained by the fluorescent antibodies. Records were kept of the background staining and the number of bacilli observed using coded smears.

Exact reproducibility was not obtained but the results of parallel investigations always showed the same trends.

MATERIALS

Substrate. The *M. tuberculosis* strain (SSC 8[illegible]1) was used as substrate in all the FAT studies in this section.

Antiserum. The homologous serum (designated Tub III in Part B) was collected after four consecutive injections; the FAT titre was 3100.

METHODS AND RESULTS

Table 1 shows the *effect of various fixation methods* using cleaned uncoated slides and slides cleaned and precoated with gelatine (see below), keeping all the other conditions in the test constant.

As will be seen from the table, the influence of the fixation method did not vary to any extent. Both heat fixation and chemical fixation were usable and combinations of heat and chemical fixation offered no advantages. However, slides precoated with gelatine showed more and generally more brilliant bacilli and a less prominent background than uncoated but cleaned slides. On the basis of these results precoated slides and the most promising of the fixation methods were used in the subsequent experiments.

Table 2 shows the *effect of incubation temperature* (22° C and 37° C) in the FAT with antiserum and normal rabbit serum, using the five best fixation methods from Table 1. There were no definite differences between the readings at the two temperatures. However, there was a tendency towards more pronounced background staining with normal rabbit serum in slides incubated at 37° C.

In this experiment use of acetone or pyridine fixation seemed advantageous and little if any difference between the two methods was seen. However, as pyridine releases unpleasant and even dangerous vapours, that chemical was omitted from subsequent experiments.

Table 3 shows the *effect of pH* in the FAT using precoated slides, acetone as fixative and an incubation temperature of 22° C. It will be seen that a broad pH optimum was found, *viz.* in the range 7.20 to 7.50. The results with normal rabbit serum show that at higher pH values

TABLE 1

Effect of Fixation Method on the FAT Using Anti M Tuberculosis Serum and Smears from the Homologous Strain

Fixation method	Slides cleaned but not coated with gelatine: Specific staining	Background	No of bacilli	Slides cleaned and coated with gelatine: Specific staining	Background	No of bacilli
1 Oven at 80 C overnight	3	+++	+	3-4	++	+++
2 *Oven at 65 C for 2 hours*	4	+++	+	4	++	+++
3 *Hot plate at 65 C for 2 hours*	4	+++	+	4	++	+++
4 Methanol for 15 minutes	3	++	+	4	+	+++
5 *Ethanol for 15 minutes*		++	+	4	++	+++
6 *Acetone for 15 minutes*	4	++	+	4	++	+++
7 10% formaldehyde for 15 minutes	4	+++	+	3	+++	+++
8 *Pyridine for 15 minutes*	4	++	+	4	++	+++
9 Isopropanol for 15 minutes	3	+++	+	4	++	++
10 5% phenol for 15 minutes	3	+++	+	3-4	+++	++
11 Method 2 + 4	3	++	(+)	3	++	+++
12 Method 2 + 5	4	+++	+	3-4	++	+++
13 Method 2 + 6	4	+++	+	4	++	+++
14 Method 2 + 7	4	++	++	3	+++	++
15 Method 2 + 8	3	++	++	3-4	+++	+++
16 Method 2 + 9	4	++	++	4	++	+++
17 Method 2 + 10	3 4	+++	++	4	+++	+++
18 Method 4 + 2	4	+++	+++	3 4	+++	++
19 Method 5 + 2	3 4	+++	++	4	+++	+++
20 Method 6 + 2	3	+++	+	3-4	+++	+++
21 Method 7 + 2	2-3	+++	(+)	2-3	+++	+++
22 Method 8 + 2	3	+++	+	3-4	+++	+++
23 Method 9 + 2	3 4	+++	++	4	+++	++
24 Method 10 + 2	3	+++	++	3	+++	+++

Fixation methods 1-3 = heat fixation 4-10 = chemical fixation 11-17 = heat fixation followed by various chemical fixation methods 18-24 = chemical fixation followed by heat fixation

Intensity of specific staining Code 0 - 4

Intensity of background staining + - +++

No of bacilli (+) - +++

The fixation methods in italics were used in subsequent experiments

there was a tendency to more intense staining of the bacilli and with both sera there was a more pronounced background colour In subsequent experiments a pH value of 7 35 was used

Additional conditions investigated included the *molarity* (μ = 0 15 was found superior to μ = 0 30 and μ = 0 07) and the *ionic composition of buffers* (0 15 M sodium chloride 0 01 M phosphate was found superior to 0 075 M sodium chloride 0 075 M phosphate and 0 15 M phosphate)

TABLE 2

Effect of Incubation Temperature on the FAT using Normal Rabbit Serum and Anti M Tuberculosis Serum as Middle Layer in Coons Indirect Technique All Smears Precoated with Gelatine

Fixation method	Normal rabbit serum Specific staining	Back ground	No of bacilli	Antiserum Specific staining	Back ground	No of bacilli
Incubation Temperature 22 C						
2	1	0	+++	4	++	+++
3	1	0	+++	4	++	+++
5	1-2	0	+++	4	+	+++
6	1	0	+++	4§	++	+++
8	1	0	+++	4	++	+++
Incubation Temperature 37 C						
2	1	+	+++	4	++	+++
3	1-2	+	+++	4	++	+++
5	1	0	+++	4	++	+++
6	1-2	+	+++	4§	+	+++
8	1	+	+++	4§	++	+++

Method 2 = oven at 65 C for 2 hours Method 3 = hot plate at 65 C for 2 hours Method 5 = ethanol for 15 minutes Method 6 = acetone for 15 minutes Method 8 = pyridine for 15 minutes

§ Bacilli very brilliant

For explanation of symbols see Table 1

TABLE 3

Effect of Increasing pH on the FAT Using Anti M Tuberculosis Serum and Normal Rabbit Serum against M Tuberculosis Smears Precoated Slides Acetone Fixation and Temperature 37 C

pH	Normal rabbit serum Specific staining	Back ground	No of bacilli	Antiserum Specific staining	Back ground	No of bacilli
6.80	1	++	+++	2	+	+++
6.90	2	++	+++	3	+	+++
7.00	1	+++	+++	2	+	+++
7.10	1-2	++	+++	3	+	+++
7.20	1-2	++	+++	4	++	+++
7.30	1-2	++	+++	4	++	+++
7.40	2	++	+++	4	++	+++
7.50	2	+++	+++	4	++	+++
7.60	2	+++	+++		+	+++

For explanation of symbol see Table 1

FINAL FAT TECHNIQUE

On the basis of the results of the methodological study the following technique was used in all subsequent experiments

1 Slides for microscopy (Socorex®) were rinsed in sulphuric acid dichromate neutralized in ammonium hydroxide rinsed in ion exchanged water and kept in ethanol until use

2 The cleaned slides were coated with 0 5 per cent gelatine air dried fixed in 5 per cent formaldehyde for 10 to 15 minutes rinsed in ion-exchanged water and air dried
3 Colonies from tubes with Lowenstein Jensen medium were emulsified in buffered saline (BS = 0 15 M saline 0 01 M phosphate pH 7 35) A drop of the suspension was placed on the coated slide which was air dried before fixation in acetone for 15 minutes at room temperature (about 2? C) The acetone was evaporated from the slides under a fan the process being continued for at least 30 minutes after the disappearance of any visible acetone

Indirect Technique

4 A drop of antiserum was placed on the smear and distributed evenly with a wooden pin
5 The slides were incubated in moist chamber for 30 minutes at room temperature
6 Th slides were rinsed in BS for 10 minutes and the surplus fluid removed from around the smear by means of a paper tissue
7 A drop of diluted goat anti rabbit globulin conjugated with FITC was placed on the smear and distributed
8 Incubation as mentioned under 5
9 Rinsing as mentioned under 6
10 The slides were mounted with ether rinsed coverslips in glycerol buffered at pH 9

Direct Technique

A drop of antiserum conjugate was placed on the smear and distributed as mentioned under 4 above The subsequent stages were as in points 5 6 and 10 above

Conjugation of antisera with FITC was performed according to Cherry et al (5)

Readings were made as described previously (1) and the results recorded as Codes 0 to 4 (?) Only results designated as Codes 3 and 4 were regarded as positive All readings were performed blindly with coded slides

PART B

IMMUNIZATION SCHEDULES SERUM PRODUCTION AND SPECIFICITY OF ANTISERA

The infection of rabbits with *M bovis M tuberculosis* and BCG causes three different immunological situations With *M bovis* a virulent infection can be provoked and on the basis of previous experience we used anti *M bovis* sera collected early in the infection period (1) A more prolonged course was necessary for the production of anti *M tuberculosis* and especially anti BCG sera

MATERIALS

Bacterial strains for serum production The strain of *M bovis* (E 4584/64) was the same as that used in previous investigations (1) The *M tuberculosis* strains (SSC 8 1 SSC 856 SSC 899) were selected on the basis of preliminary results which showed serological difference between them

M bovis var BCG = ATCC 19274

Bacterial strain for specificity determination The 13 strains used form part of Series 1 issued by the International Working Group on Mycobacterial Taxonomy (see Table 5 below)

TABLE 4

Immunization Schedule for Production of Antisera

Designation of sera	Strain used for immunization	No of injections	Time of injections (days after onset)	Inoculum	Time of bleeding (days)	Titres of sera	Titres of final pools of sera
Anti *M bovis* I	E 4584/64	1	0	1 ml i.v.	14	100 – 316	316 †
Anti *M bovis* II	E 4584/64	8	0 3 7 10 14 17 21 24	1–2 ml §	28	< 10	< 10
Anti BCG	ATCC 19274	4	0 7 14 21	0 1–1 ml	28	316	316
Anti *M tuberculosis* I	SSC 821	1	0	1 ml i.v.	28	100 – 1000	316
Anti *M tuberculosis* II	SSC 856	3	0 7 14	0 1 ml i.v. 0 1 ml i.v. 1 ml s.c.	32	100 – 316	316
Anti *M tuberculosis* III	SSC 891	4	0 7 14 21	0 1–1 ml	28	1000 – 3160	3160 †
Anti *M tuberculosis* IV	SSC 829	3	0 64 129	0 1 ml i.v.	180	316 – 1000	1000

Sera used in the indirect technique † Sera used in the direct technique § killed bacilli

METHODS AND RESULTS

Immunization and production of serum The strains were grown in Dubos liquid medium with Tween 80 Before inoculation the cultures were adjusted to an optical density of 0 1 in Coleman Junior spectrophotometer at 595 nm using 10 mm cuvettes Given volumes of these suspensions were inoculated into groups of four rabbits according to the immunization schedule shown in Table 4 During the immunization period blood samples were drawn at intervals from the ear vein and the FA titres against homologous strains were estimated At the end of the immunization periods the animals were bled by heart puncture and the serum collected Phenol and EDTA were added to final concentrations of 0.5 per cent and merthiolate to 0 01 per cent The sera were stored at +4 C or —70 C.

Anti M bovis Sera

Anti M bovis I serum (Bov I) was produced by a single injection of viable bacilli Among the four sera obtained two showed a FA titre of 316 and two a titre of 100 The first named sera were pooled and the FA titre of the pool was 316

Anti M bovis II serum was collected following hyperimmunization with phenol killed bacilli None of the sera gave positive FA reactions

The *BCG* immunization consisted of four consecutive injections with viable bacilli Among the four rabbits used two died following the third injection and one following the fourth The surviving rabbit was bled and the FA titre of its serum was 316

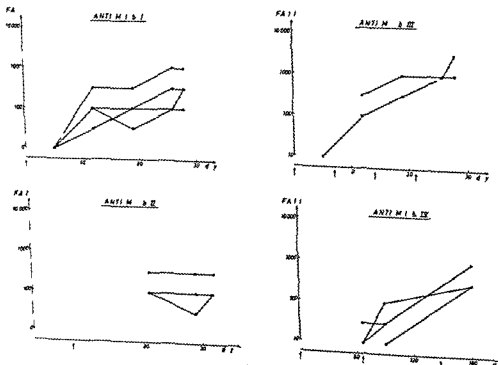


Fig 1

Titre curves of sera from rabbits injected with M *tuberculosis* according to immunization schedule shown in Table 4 Arrows indicate time of injection Titre is defined as the reciprocal of the last dilution still giving positive reading in the indirect FAT

TABLE 5

Comparison of Specificity of Different Sera in the Indirect FAT

Substrate		Serum					
Designation	Strain No	Bov I	BCG	Tub I	Tub II	Tub III	Tub IV
Strains used for serum production							
M. bovis	I 4584/64	+	+	—	—	+	+
M. bovis (BCG)	ATCC 19274	+	+	+	+	+	+
M. tuberculosis	SSC 821	+	+	+	+	+	+
M. tuberculosis	SSC 856	+	+	+	+	+	+
M. tuberculosis	SSC 829	+	+	+	+	—	+
Series issued by International Working Group on Mycobacterial Taxonomy							
M. avium	ATCC 15769	—	—	—	—	+	—
M. intracellularis	ATCC 13950	—	—	—		—	—
M. kansasii	ATCC 12478		—	—	—	—	—
M. marianum	ATCC 19075	—		—	—	—	—
M. bovis	ATCC 19210	—	—	—	—	+	—
M. bovis (BCG)	ATCC 19274	+	+	+	+	+	+
M. marinum	ATCC 927	—		—	—	—	—
M. fortuitum	ATCC 6841	—	—	—	—	—	+
M. smegmatis	ATCC 14468	—		—	—	—	+
M. phlei	ATCC 19249			—	—	—	—
M. vaccae	ATCC 15483			—	—	—	—
M. xenopei	ATCC 19970			—	—	—	—
M. microti	ATCC 19422	—		—	—	—	—

Anti M. tuberculosis Sera

Four different methods were employed for the production of anti M. tuberculosis sera using viable bacilli. Titre curves during immunization are shown in Fig. 1.

Anti M. tuberculosis serum (Tub I) was produced by a single intravenous injection. The four sera had FA titres of 1000, 316, 316 and 100 respectively. The first two of these were pooled and the resulting titre was 316.

Anti M. tuberculosis II serum (Tub II) was produced by two consecutive intravenous injections followed by one subcutaneous injection. Among the four sera two had a FA titre of 316 and two a titre of 100. The first two sera were pooled and the resulting titre was 316.

Anti M. tuberculosis III serum (Tub III) was produced by four consecutive injections. Among the four rabbits used two died following the fourth injection. The two surviving rabbits had FA titres of 3160 and 1000 and the titre of the pooled sera was 3160.

Anti M. tuberculosis IV serum (Tub IV) was produced by three consecutive intravenous injections of small doses at long intervals. Among the four sera one had a FA titre of 1000 and three a titre of 316 after 180 days. The first named serum was used in the specificity comparisons.

Specificity Determination of Sera

Table 5 shows the results of the FAT using the different mycobacterial strains and the six sera diluted to the penultimate titration stage.

In this experiment, the sera designated Tub I and Tub II reacted with BCG and M. tuberculosis exclusively. Serum Tub III reacted also with *M. avium* and *M. bovis*. Serum Tub IV reacted with *M. fortuitum* and *M. smegmatis*. When all the sera were tested repeatedly against other *M. fortuitum* strains, they all gave positive reactions with a frequency of about 10 per cent.

These results were decisive for the selection of the two anti *M. tuberculosis* sera (Tub I and Tub II) for use in the indirect technique in Part C.

PART C

SEROTYPING WILD STRAINS OF M BOVIS AND M TUBERCULOSIS

For the practical application of the FAT in the laboratory diagnosis of tuberculosis it is essential to have a knowledge of the serological relationship between mammalian mycobacteria Preliminary results showed some differences between *M bovis* and *M tuberculosis* and to a certain degree also within the latter species A number of wild strains isolated from Danish patients with tuberculosis were therefore tested against some of the sera mentioned in Part B

MATERIALS

Bacterial strains tested by the sera (substrate) 104 strains of M tuberculosis and 42 strains of *M bovis* were selected from strains isolated at the Tuberculosis Department of Statens Seruminstitut Copenhagen in 1965 Before testing they were kept in tubes with Löwenstein-Jensen medium at about 22 C for one to four months Smears were made direct from these stock cultures

Antisera The sera designated Bov I BCG Tub I and Tub II in Part B were used in the indirect technique and conjugates of Bov I and Tub III in the direct technique

METHODS

For details of the methods used see Part A

RESULTS

Tables 6 and 7 show the results of serotyping 146 strains (104 *M tuberculosis* and 42 *M bovis*) using the indirect fluorescence technique

Table 6a shows the distribution of the results when Tub I and Tub II were used as test sera Among the 17 inconsistent results with the *M tuberculosis* strains four were read as — Tub I + Tub II This distribution has a p value of 4 9 per cent in a binomial distribution

TABLE 6

Distribution of Results when 104 M tuberculosis *and 42* M bovis *Strains Tested against* M tuberculosis *Antisera (a) and* M bovis *and BCG Antisera (b) in the Indirect FAT*

	Antisera	Strains: *M tuberculosis*	Strains: *M bovis*
(a)	— Tub I — Tub II		30
	— Tub I + Tub II	4	4
	+ Tub I — Tub II	13	5
	+ Tub I + Tub II	87	3
(b)	— Bov — BCG	3	3
	— Bov + BCG	8	4
	+ Bov — BCG	8	4
	+ Bov + BCG	85	31

with $n = 17$ and $p = 0.7$ (The statistical evaluation of such distributions has been described previously (3).) The negative frequencies with the two sera are about 9 and 17 per cent respectively. In the *M. bovis* material, about 80 per cent of the strains did not react with the sera.

Table 6b shows the corresponding results when Bov I and BCG were used as test sera. The two sera reacted equally well with negative frequencies of 10 and 16 per cent in the *M. tuberculosis* and *M. bovis* material respectively.

Table 7a shows the distributions when the reactions with the two anti-*M. tuberculosis* sera are regarded as one double reaction and where only one positive reading permits notification of a result as positive. Correspondingly the reactions with Bov I and BCG are regarded as one double reaction. In the *M. tuberculosis* material anti-*M. tuberculosis* serum and anti-*M. bovis* serum reacted equally well. However, in the *M. bovis* material the superiority of anti-*M. bovis* serum as regards staining efficiency is significant.

TABLE 7

Evaluation of Results from Table 6

	Antisera	Strains: *M. tuberculosis*	Strains: *M. bovis*
(a)	− Tub − Bov	1	3
	− Tub + Bov	4	27
	+ Tub − Bov	2	0
	+ Tub + Bov	97	12
(b)	− Tub − Bov	7	10
	− Tub + Bov	15	29
	+ Tub − Bov	12	1
	+ Tub + Bov	70	2

Two reactions with *M. tuberculosis* antisera are regarded as one test and two reactions with *M. bovis* antisera as one test. One positive result permits positive notification in (a) and both results must be positive for positive notification in (b).

Table 7b shows the corresponding results with the double reactions when positive readings from both reactions are necessary for a positive notification.

Tables 8 and 9 show the results of testing the 42 *M. bovis* strains by the direct immunofluorescence technique.

Table 8a shows the distribution of results when the strains are tested twice against the anti-*M. tuberculosis* conjugate. The average negative frequency of the reactions is 67 per cent and the distribution of inconsistent results (viz. 6 and 10) may be fortuitous. Table 8b shows the corresponding results when the strains are tested twice against anti-*M. bovis* conjugate. The negative frequency is about 12 per cent for each reaction.

Table 9a shows the results when the two consecutive reactions with the anti *M. tuberculosis* conjugate and the anti *M. bovis* conjugate are regarded as double reactions and where one positive reading per double reaction permits notification of a result as positive. In Table 9b both readings in the double reaction must be positive for a positive notification. In both cases the results demonstrate the superiority of *M. bovis* conjugate for staining *M. bovis*.

Finally mention should be made of a preliminary series of investigations where 485 strains of *M. tuberculosis* were tested once against the *M. tuberculosis* conjugate. The negative frequency in that series was 9 per cent.

TABLE 8

Distribution of Results when 42 M. bovis Strains Tested Twice against Anti M. tuberculosis Conjugate (a) and anti M. bovis Conjugate (b) in the direct FAT

	Conjugate	*M. bovis* strains
(a)	— tub 1 — tub 2	18
	— tub 1 + tub 2	6
	+ tub 1 — tub 2	10
	+ tub 1 + tub 2	8
(b)	— bov 1 — bov 2	0
	— bov 1 + bov 2	5
	+ bov 1 — bov 2	5
	+ bov 1 + bov 2	32

TABLE 9

Evaluation of Results from Table 8

	Conjugate	42 *M. bovis* strains
(a)	— tub — bov	0
	— tub + bov	18
	+ tub — bov	0
	+ tub + bov	24
(b)	— tub — bov	9
	— tub + bov	25
	+ tub — bov	1
	+ tub + bov	7

Two reactions with anti *M. tuberculosis* conjugate are regarded as one test and two reactions with anti *M. bovis* conjugate as one test. One positive result permits positive notification in (a) and both results must be positive for positive notification in (b).

DISCUSSION

The aim of this study was to examine the specificity of circulating antibodies produced by infection with viable tubercle bacilli. Since rabbits were used for antibody production a virulent infection due to

M bovis could be provoked and antibodies obtained early in the infection could be examined on the lines of previous investigations (1 2). A corresponding production of antisera against *M tuberculosis* and BCG was not thought to be possible and different immunization schedules were therefore adopted in order to feign the multiplication of bacilli in the virulent infection. It was possible by repeated injections of viable bacilli to produce antibodies against *M tuberculosis* and BCG in rabbits. The technical problems involved the risk of death of the animal within one to two days after the later injections and the possibility of obtaining an antibody response against minor antigens due to the prolonged immunization schedule. One of the sera used was produced by a single injection of viable bacilli and showed a high degree of type specificity though a long immunization period was necessary.

The specificity of the antibodies obtained against different mycobacterial strains was reasonably good as will be seen from Table 5. Anti *M tuberculosis* III gave reactions with the *M avium* type strain and consequently was not used in the indirect technique. However on account of its high titre this serum was used in the direct technique. The other sera employed gave no cross reactions with such important bacteria as *M avium*, *M intracellularis*, *M kansasii* and *M xenopei*, but all of them reacted occasionally with *M fortuitum*. Thus other types of sera might be necessary for use in routine laboratory typing.

The fluorescence antibody technique used was based on the results of a methodological study performed in an attempt to decrease the number of false negative results. An important detail of the technique is the use of precoated slides which during the subsequent manipulations retain the fixed bacilli better than cleaned but uncoated slides. Pyridine and acetone gave the best results but because of the unpleasant vapours liberated by pyridine acetone was preferred. The final technique used in the subsequent typing still gave a certain number of false negatives which necessitated a statistical evaluation of the results. In routine typing the examination of a number of parallel slides would be required.

In serotyping the *M tuberculosis* and *M bovis* strains the frequency of negative results and the distribution of the inconsistent results were decisive for the evaluation. All the sera examined showed the same trend as regards staining property. Anti *M bovis* sera (including BCG) stained *M tuberculosis* and *M bovis* while anti *M tuberculosis* sera generally stained *M tuberculosis* only.

The opinions of various workers differ concerning serological differentiation between mammalian mycobacteria. Most workers do not distinguish between *M tuberculosis* and *M bovis* but a few reports present possibilities for differentiation similar to those found in this study. Schaefer (6) working with polysaccharide and protein antigens from culture filtrates found a protein antigen in the filtrate from bovine

strains that was not present in filtrates from BCG or human strains. Later he reported the same possibility of differentiation with a hydrochloric acid extract from whole bacilli (7). By means of immuno-electrophoresis and immunodiffusion *Castelnuovo et al.* (4) revealed a difference between *M. tuberculosis* and *M. bovis*. The latter possessed an antigen that was not found in the former or in BCG. Furthermore, another antigen found both in *M. tuberculosis* and *M. bovis* was lacking in BCG.

The difference found in the present study seems to be parallel to that described by *Schaefer* and might be explained in the same way, *viz.* the existence of an antigen common to *M. bovis* and *M. tuberculosis* and an antigen specific for *M. bovis*. However, we found that BCG was able to induce antibody production against the specific *M. bovis* antigen.

For the practical application of these results it is necessary to place *M. bovis* in one serotype and *M. tuberculosis* in another, so far as immunofluorescence work is concerned. In the material examined we found no definite evidence of serological variations within the two species. The different strains of *M. tuberculosis* employed in serum production were selected on the basis of preliminary results where variations were found. As mentioned in Part C, the difference between the two anti-*M. tuberculosis* sera was on the borderline of statistical significance and thus the serological variation, if any, must be slight. The Bov I and BCG sera showed a slightly higher frequency of negative results with *M. bovis* than with *M. tuberculosis*. This phenomenon might be explained by a higher degree of variation in the *M. bovis* strains. However, the conditions for the FAT were established in experiments with *M. tuberculosis* and those conditions might be less favourable for *M. bovis* strains.

If the immunofluorescence technique is to be employed in routine laboratories for the specific staining of tubercle bacilli, anti-*M. bovis* serum should be used in order to stain both *M. tuberculosis* and *M. bovis*, and anti-*M. tuberculosis* sera stained strains of *M. tuberculosis* with equal efficacy, but that anti-*M. bovis* serum was significantly more suspected tuberculosis patients, smears from *M. tuberculosis* should be used as substrate or, alternatively, smears from both species must be employed.

SUMMARY

Circulating antibodies in rabbits infected with *M. bovis* and *M. tuberculosis* were examined from the point of view of their specificity in the fluorescence antibody test (FAT). Statistical evaluation of the results of serotyping strains from tuberculous patients showed that anti-*M. bovis* and anti-*M. tuberculosis* sera stained strains of *M. tuberculosis* with equal efficacy, but that anti-*M. bovis* serum was significantly more effective in staining *M. bovis* strains.

The conditions for the IAT were examined in a methodological study the results of which are included in this paper

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STUDIES ON THE QUANTITATION OF LYMPHOCYTE RESPONSE *IN VITRO*

By

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Received 11 xii 68

Submitted to suitable culture conditions blood lymphocytes can be induced to proliferate by several stimuli such as unspecific mitogens antigens against which the lymphocyte donor is sensitized antibodies against determinants on the lymphocyte surface and transplantation antigens both in the form of intact cells and as cell free preparations

The present investigation is based on a method of culture which allows good cell survival and early occurrence of significant thymidine incorporation in the stimulated cultures Three kinds of stimulation have been used 1) phytohaemagglutinin (PHA) as an example of an unspecific mitogen 2) purified tuberculin and 3) mixed cultures of allogeneic cells

Several investigators (2 3 5 9 11) have stressed the importance of standardizing culture conditions and assay methods Lack of reproducibility has been a major problem in quantitation of the lymphocyte response by means of morphological methods such as the counting of transformed cells and mitoses or of labelled cells in autoradiographs Therefore a systematic study of the rate of thymidine incorporation during 7 8 days of culture was undertaken A rapid and accurate quantitation was obtained by means of a modification of the assay method proposed by *Mosedale & Park* (8)

It has been shown that the maximal thymidine uptake in cultures of the same type may occur on different days Hence determination of the thymidine incorporation at a fixed time when the peak response is supposed to occur may be misleading Fortunately however it appears that the response is best quantitated by measurements during the first days of proliferation *i e* before the peak response occurs

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MATERIAL AND METHODS

Preparation of the Cultures

50 ml of blood from normal adults is defibrinated for 10 minutes in an Erlenmeyer flask containing 10 1 glass beads and mixed with equal volumes of the following solution 7 parts of TC 199 (Glaxo) containing penicillin and streptomycin and 3 parts of dextran 6 per cent in 0 9 per cent sodium chloride (Intradex Glaxo) After sedimentation for 30 60 minutes at 37 C 50 60 ml of supernate can be removed

The sediment is centrifuged (1000 g 15 minutes) for preparation of cell free medium

The number of lymphocytes and granulocytes in the leucocyte suspension is determined by phase contrast microscopy using an ordinary haem cytometer The concentration of lymphocytes is adjusted to 1 million per ml either by concentration of the suspension by centrifugation (200 g 10 minutes) or by dilution with cell free medium Normally it is possible with this method to obtain 40–80 million lymphocytes with a granulocyte content of 0 5–2 million per million lymphocytes

The suspension is transferred to culture vials usually 2 or 5 ml per vial Brown screw capped medicine bottles have been used delivered a eptic from Pl3t Manufaktur Limmared Sweden The volume of the vial is 10 ml the internal diameter being 19 mm

The gas phase of the vials is 5 per cent CO in atmospheric air The cultures are maintained at 37 C.

Stimulation of the Cultures

Reconstituted PHA (Burroughs Wellcome) or purified tuberculin 0 02 mg per ml (Statens Seruminstitut Copenhagen) is added to the cultures in the concentrations stated Stimulation with tuberculin was investigated in cultures from person with a positive tuberculin skin test

Mitogen is added to the remaining cell free medium in the same concentration as in the cultures and it is stored at 4 C for later change of medium

Mixed cultures are prepared using equal volumes of leucocyte suspension from two unrelated persons total volume 2 or 5 ml per culture

Change of Medium

Unless otherwise stated change of medium is performed on days 2 4 and 6 The upper half of the culture medium is removed without disturbing the cells at the bottom of the vial and is replaced by an equal volume of cell free medium prewarmed to 37 C The cultures are re flushed with 5 per cent CO in air before the caps are closed

Harvest of the Cultures

The cultures are terminated at 24 hour intervals After a careful resuspension of the cells 1500 µl is transferred to new culture vials containing radioactive tracer

The remaining suspension is used for viability testing and smears

After incubation with tracer for 240 minutes at 37 C the cells are resuspended and 1000 µl is collected on Whatman glass fibre filters type GF/C, diameter 12 8 mm mounted in Millipore filterholders connected to a water suction system

The rest of the labelled suspension is used for autoradiography

The cell deposit on the filter is treated successively with 60 ml of isotonic saline 40 ml of ice cold trichloroacetic acid and 40 ml of methanol

In preliminary experiments the filters were counted directly with the cell coated surface upwards in 15 ml of dioxan scintillator (naphtalene 60 g methanol 100 ml ethylene glycol 20 ml PPO 5 g dimethyl POPOP 0 3 g made up to 1 litre with dioxan)

In all subsequent experiments the active cell material on the filters has been digested with hyamine (10) The dried filters are transferred to counting vials and 1000 µl of hyamine (Packard) is added After 24 hours in the dark at 37 C 15 ml of toluene scintillator (PPO 5 g + dimethyl POPOP 0 3 g per litre toluene) is added The samples are counted in a Packard Tri Carb Liquid Scintillation Counter

Type 314 EX 1 to a counting error below 1 per cent All results are expressed as counts per minute per 1 million lymphocyte of the initial culture The background counts are subtracted from all data

Tracer

Thymidine 2 ^{14}C (specific activity 60 5 mCi/mM) 0 1 μCi per ml culture, Uridine 2 ^{14}C (specific activity 58 1 mCi/mM) 0 1 μCi per ml culture and DL Leucine 2 ^{14}C (specific activity 51 mCi/mM) 1 0 μCi per ml of culture have been used All tracers have been purchased from The Radiochemical Centre Amersham England

Viability Counting

Viability counting is performed by direct counting of living and dead cells under phase contrast illumination in an ordinary haemocytometer The dead cells are characterized by cytoplasmic oedema and nuclear pyknosis No results of the viability counts during the first 3 days are given as the presence of polymorphs disturbs the counting during this period

RESULTS

Assay Method

When assaying the incorporation of ^{14}C labelled tracers the cells can be deposited on a glass fibre filter and counted directly in a dioxan scintillator (8) With this method the activity remains localized to the filters (10) It is however possible to dissolve the active cell material on the filters with hyamine In the case of non hyamine treated filters the highest counting efficiency was obtained using dioxan scintillator while hyamine treated samples were counted with the greatest efficiency using toluene scintillator

It is seen (Table 1) that the hyamine treated filters—*in spite of* hyamine quenching—constantly gave higher counts than the non hyamine treated filters After turning the non hyamine treated *filters with* the cell side downwards the count rate decreased by 5–10 per cent When the non hyamine treated filters were transferred *to new* counting vials the activity was transferred with the filters *while* the activity remained in the counting vials after removal of the hyamine treated filters showing that the active cell material was dissolved

Both methods showed a linear relationship between the amount of labelled cells collected on the filters and the activity found and both methods may therefore be used The hyamine method which was worked out primarily for counting of ^{3}H labelled tracers (10) was preferred for counting of ^{14}C labelled tracers *too* due to the higher counting efficiency and the independence of geometrical factors

Change of Medium

The survival and proliferation of the cells are improved considerably by change of medium Fig 1 shows the thymidine incorporation and number of living cells in mixed cultures when in one set of vials change of medium had been performed on days 2 4 and 6 while in the control set only flushing of the cultures with 5 per cent CO_2 in air was

TABLE 1

Comparison between Assays of Non hyamin Treated and Hyamin Treated Filters

Culture No	µl on filter	Assay method	c p m	c p m after turning the filter with cells downwards	% fall	c p m after removal of filter	% fall	c p m in new vials
	500	H	3027			2983	1.5	1/3
	500	U	2924	2602	9	1		2731
	1000	H	6274			6176	1.5	60
	1000	U	5700	5319	6.5	3		5213
3/71	1500	H	9447			9391	0.7	343
	1500	U	8438	7746	8	4		8537
	2000	H	12927			12780	1.1	383
	2000	U	11495	10761	6.5	2		10533
	500	H	4366			4382	0	163
	500	U	4213	3800	10	24		3845
	1000	H	8961			9006	0	137
	1000	U	8271	7780	6	199		7655
37/2	1500	H	13459			13193	2.0	176
	1500	U	12012	11430	5	40		11146
	2000	H	18262			18176	0.5	333
	2000	U	14010	15330	10	9		15461

H Hyamin treated filters counted in 15 ml of toluen scintillator
U Non hyamin treated filters counted in 15 ml of dioxan scintillator

Two different pools of PHA stimulated cultures were harvested on filters in the amounts indicated 24 hours after addition of scintillation fluid the non hyamin treated filters were turned with the cell side downwards 48 hours after the addition of scintillation fluid all filters were removed from the original counting vials and transferred to new vials (the orientation of the filters in the new glasses was not controlled)

done on the same days It is seen that due to a higher proliferative activity twice as many living cells were found in the cultures on day 8 when the medium was changed as compared with control cultures Surprisingly daily change of medium did not further improve neither cell survival nor thymidine incorporation *Change of medium on day 3* only gave less than optimal activity These results held true in both PHA and tuberculin stimulated cultures

The culture medium was diluted by 30 per cent Intradex To investigate whether the need for change of medium was due to the decreased concentration of nutritive factors in the media PHA and mixed culture experiments were performed in which the cells were isolated by centrifugation and cultured in medium with and without *Intradex* No significant difference was observed implying that culturing of cells in undiluted medium could not substitute for change of medium

The addition of 2 5 or 5 mg of glucose to the cultures instead of change of medium on days 2 4 and 6 did not increase the activity above that obtained in cultures in which the medium was not changed

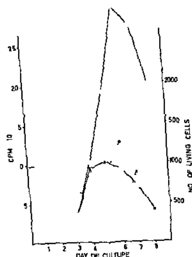


Fig 1

A comparison between mixed cultures with and without change of medium
O Change of medium
X No change of medium
Dotted lines Number of living cells
Fully drawn lines Thymidine incorporation

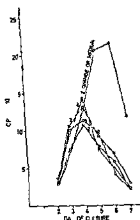


Fig 2

Mixed cultures Increasing volumes of medium have been added to different cultures while the medium has been changed in one culture
See text. The numbers indicate the volume of culture in ml

In stimulated cultures the pH of the medium decreases To investigate whether the effect of the change of medium was due to a partial normalization of the pH in the cultures triplicate mixed cultures were set up To one series bicarbonate was added on days 2 4 and 6 restoring neutral pH as estimated by the colour of the indicator This resulted in a thymidine incorporation higher than that in the control cultures but the activity did not reach the same level as in the cultures in which change of medium was performed

To investigate the possibility that the medium might be exhausted due to an excessive concentration of cells the following experiment was made Liquid volumes of leucocyte suspensions from two donors were mixed and dispensed into 5 groups of 6 culture vials each containing 2 ml To the 2nd 3rd and 4th group was added 1 2 and 3 ml of cell free medium respectively so that the final lymphocyte concentration in the 5 groups were 1 00 0 67 0 50 0 40 and 1 00 millions per ml Change of medium was made in the cultures of the 5th group only while the remaining cultures were flushed with 5 per cent CO in air on the same days It is seen (Fig 2) that no significant differences were found in the cultures in which no change of medium was performed irrespective of cell concentration while the cultures in which the medium was changed showed much higher and longer lasting thymidine incorporation

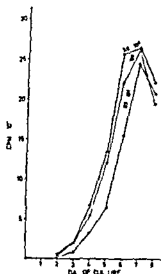


Fig 3

A comparison between mixed cultures with different numbers of cells per culture See text

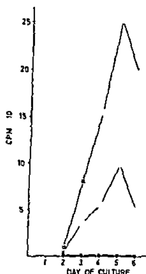


Fig 4

Cultures from a tuberculin hypersensitive person which have been stimulated with different concentrations of purified tuberculin

X–X 0 01 μg per ml

□–□ 0 2 μg per ml

■ ■ 1 μg per ml

The results in Fig 2 also imply that the height of the medium in the cultures in which no change of medium has been performed was of no importance for the thymidine incorporation The same finding has consistently been made in cultures in which the medium was changed

Number of Cells per Culture

To evaluate the significance of the cell density at the bottom of the culture vials mixed culture experiments were made in which the cell concentration was kept constant *i e* 0 5 million per ml while the number of lymphocytes per culture were 0 5 2 and 3 million respectively (Fig 3) It is seen that no significant difference in thymidine incorporation between cultures containing 2 and 3 million lymphocytes was found while the thymidine incorporation in vials containing 0 5 million lymphocytes was delayed compared to the cultures with higher cell density

In other mixed culture experiments and in experiments with tuberculin stimulation in which the lymphocyte concentration was 1 million per ml no difference in the thymidine incorporation was found in vials containing 2 and 5 million cells

On the basis of these experiments it is not possible to determine the optimal density of cells per square unit (7) as the bottom of the vials

is slightly curved so that the cells settle with the highest density along the periphery

Concentration of PHA and Purified Tuberculin

The optimal concentration of PHA was found to be 0 015 ml per ml of culture

The optimal concentration of purified tuberculin varied from individual to individual and must be determined in each case Fig 4 shows a tuberculin titration In other experiments a similar small difference between the optimal concentration and the toxic concentration was found The optimal concentration varied between 0 2 and 1 5 μg of purified tuberculin per ml of culture

Protein RNA and DNA Synthesis

In PHA stimulated cultures significant RNA and protein synthesis is seen within one hour after the initiation of culture whereas DNA synthesis commences at 24 hours (6) The time course of RNA protein and DNA synthesis in antigen stimulated cultures is shown in Figs 5a and 5b The observed parallelism between the incorporation of uridine leucine and thymidine was also found in cultures with weaker responses On the first day of culture significant incorporation has never been encountered

In most of the figures thymidine incorporation by the unstimulated control cultures has been omitted The results depicted in Figs 5 and 7 are however representative It is seen that the activity in the control cultures was slightly increasing towards the 8th day As a rule the thymidine incorporation in the controls was about 200–500 CPM In a few control cultures an increase to 1 000 CPM was seen on days 7 and 8 As seen from Fig 5 a considerable incorporation of leucine and uridine was found in unstimulated cultures

These results indicate that assay of DNA synthesis is the most suitable parameter for quantitation of the lymphocyte response

Reproducibility

In order to assess the variation between individual vials in mixed cultures and tuberculin stimulated cultures the coefficient of variation was calculated on the basis of 44 determinations in duplicate and 14 determinations in triplicate The result was 10 2 per cent for mixed cultures and 9 6 per cent for tuberculin stimulated cultures which gave a weighted mean of 10 0 per cent

Four out of these 58 experiments showed clearly deviating results (coefficients of variation 21 5 25 4 26 1 and 32 4 per cent) suggesting some unknown technical error If these four experiments were disregarded the coefficient of variation was reduced to 7 0 per cent 7 6 per cent and 7 2 per cent respectively

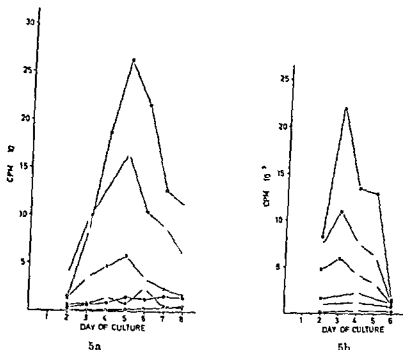


Fig 5

^{14}C Leucine ^{14}C Uridine and ^{14}C Thymidine uptake in leucocyte cultures
×–× ^{14}C Leucine ○–○ ^{14}C Thymidine ●–● ^{14}C Uridine
5a Mixed cultures 5b Cultures from a strongly tuberculin hypersensitive person stimulated by 0.2 μg purified tuberculin per ml
Three lower lines show the average activity of the unstimulated cultures

The *filter to filter variation* was estimated on the basis of 49 experiments in duplicate. The coefficient of variation was 3.3 per cent.

To get an impression of the *day to day variation* mixed cultures from the same two persons were prepared at intervals of 3 days. The result is seen from Fig 6.

Cell Death—Peak Response

A leucocyte culture is both a growing and a dying culture. Among the granulocytes the polymorphs die during the first days but the eosinophils survive until late in the culture. The number of living lymphoid cells decreases from the first day of culture at a rate which is largely determined by the culture conditions (Fig 1). When stimulation causes intensive proliferation a transitory increase in the number of living cells occurs, see Fig 7. After day 8 a rapid decrease in the number of living cells always occurs in the culture system described. Figs 1 and 7 show furthermore that the thymidine incorporation by the stimulated cultures begins to decrease at a time when the number of living cells in the cultures is still increasing, showing that thymidine incorporation is inhibited already before massive cell death occurs.

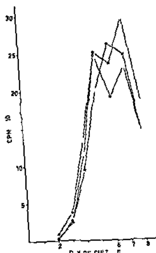


Fig 6

Mixed cultures prepared from the same two test persons with an interval of 3 days
The points represent the average of duplicate or triplicate determinations

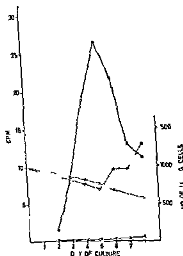


Fig 7

Thymidine incorporation and number of living cells in mixed cultures and controls
Fully drawn lines Thymidine incorporation
Dotted lines Number of living cells
O–O Mixed cultures
X–X Average of unstimulated controls

Fig 8 shows the thymidine incorporation in the experiment described in Fig 5b compared with the thymidine incorporation in cultures from a person who showed a weaker skin test reaction to purified tuberculin It is seen that the stronger skin reactor showed an early and violent response with a peak already on the 3rd day of culture while the weaker reactor showed a slower but still increasing thymidine incorporation on day 5 In other cultures stimulated by tuberculin, the peak response was found on day 4 or 5

In PHA stimulated cultures peak responses were found on day 2 or 3

In mixed cultures in which change of medium was performed (cf Figs 1 2 3 and 6) the peak response was found from day 5 to 7

DISCUSSION

When incorporation of DNA precursors is employed to quantitate the proliferative response of lymphocytes *in vitro*, the measurement is often carried out at a fixed time when the response is believed to be maximal e g on the 3rd or 4th day in PHA stimulated cultures on the 5th or 6th day in tuberculin stimulated cultures and on the 6th or 7th day in mixed cultures The present investigation shows however that the

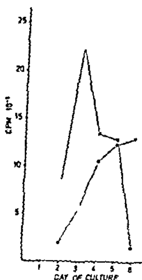


Fig 8

Thymidine incorporation in cultures from two different test persons stimulated by purified tuberculin

O-O The same person as in Fig 5b who by skin test using 0 02 µg purified tuberculin showed reddening and edema of the whole antebrachium

● ● A person who by skin test using 0 02 µg purified tuberculin showed reddening and induration in an area of 1 × 1 cm

Both cultures were stimulated by 0 ? µg purified tuberculin which in other experiments was found to be the optimal concentration for both test persons.

peak response may occur on different days when the same antigenic stimulant is applied to lymphocyte cultures from different persons

Hives (1) has recently reported improved cell survival in mixed cultures from inbred strains of rats by change of medium in the cultures In cultures of human lymphocytes too change of medium will improve cell survival and thymidine incorporation (Figs 1 and 2) The characteristic finding is that the peak response is thereby increased and it occurs later in the culture period thus demonstrating that peak response is determined not only by the proliferation of the cells but is also greatly influenced by culture conditions

Furthermore Figs 1 and 2 demonstrate that the thymidine incorporation on day 3 and 4 is identical in cultures from the same individuals whether or not change of medium has been performed suggesting that the early response is much less influenced by the culture conditions and therefore a better measure of the cellular response

In mixed cultures from inbred strains of rats and their F_1 hybrids *Wilson et al* (12) have shown that new cells are continuously entering mitosis for the first time from day 2 to day 7 and that the cells in mitosis then proceed through a successive series of divisions with a constant generation time In mixed cultures from man *Wilson et al* have found a generation time of 14 hours

The immunological significance of the finding that some cells do not start proliferation until late in the cultures has not yet been clarified. However, due to the short generation time, the response will be dominated by the cells which begin proliferation early, while cells which enter into mitosis late in culture contribute only to a small extent to the total thymidine incorporation.

That the exponential rise of the thymidine incorporation curve levels off before the peak is reached shows that some of the cells already at this time fall out of the mitotic cycle. The reason for this is presumably cell death which at least in part is due to suboptimal culture conditions.

In the quantitation of the lymphocyte response it therefore seems more reasonable to measure the thymidine incorporation during the first days of proliferation rather than the peak response. Fig. 4 shows that the optimal tuberculin concentration can be determined on days 3 and 4 as well as later in the cultures. Fig. 8 shows that a person with a violent reaction to tuberculin skin test shows an early, strong response *in vitro* in comparison to a person with a smaller degree of sensitivity and that assessment on day 5 only would have been misleading.

In mixed culture experiments the response can likewise be quantitated during the first three to four days of culture. Investigations are at present in progress using one way cultures between HL-A typed family members in order to determine the correlation between the early response *in vitro* and the degree of HL-A incompatibility.

SUMMARY

The present investigation was undertaken in an attempt to define the optimum conditions for quantitation of the lymphocyte response *in vitro*.

The cellular uptake of ^{14}C-thymidine was measured using a modification of the method proposed by *Mosedale & Parke*. Change of medium was shown to be of major importance for cell survival and proliferation.

The peak response was determined not only by the antigen concentration and the sensitivity of the cells but also by the culture conditions. The initial response was much less influenced by the culture conditions and therefore from a technical point of view was a better measure of the cellular response.

The results obtained with cultures stimulated by tuberculin in different concentrations and from persons with different degrees of tuberculin hypersensitivity suggest that the lymphocyte response may be quantitated by measuring the rate of thymidine incorporation during the first days of culture.

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GEL PRECIPITATION REACTIONS BETWEEN ALKALINE EXTRACTED RUBELLA ANTIGENS AND HUMAN SERA

By

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Received 24 xii 68

In a preliminary report (10) we have recently demonstrated two specific gel precipitation lines with rubella antigens and rubella convalescent human sera Schmidt et al have also reported rubella gel precipitation in agarose gel (11) They were able to show at least one possibly two separate lines and the reactions were found both with viral and "soluble antigens

This report describes the gel precipitation haemagglutination inhibition and complement fixation reactions of sera from rubella patients as well as of randomly selected female sera

At least three possibly four precipitation lines specific for rubella virus were demonstrated with some of these sera

MATERIAL AND METHODS

Virus The rubella virus strain employed was the RA 7/3 strain (15) It was used at the second and third passage in this laboratory

Cells BHK 21/13 S cells in suspension culture were used in antigen preparations The details of the cell culture methods have been described elsewhere (3) The cells were used at the 11th to 30th passages in this laboratory

Preparation of antigens The methods used are reported in details by *Halonen et al* (3) For the preparation of antigens the trypsinized cells were grown in suspension in a 500 ml Erlenmeyer flask with screw cap in constant spinning with a magnetic stirrer The volume of the culture was 400 ml After a preliminary growth period of 1-2 days when the number of cells was 200-400 millions per culture the pH was adjusted to neutral with 7 per cent sodium bicarbonate and the cells were inoculated with rubella virus at a multiplicity of about 0 1 PFU/cell The cells were placed in a 35 C incubator shaken at 15 minutes intervals and after one hour the suspension was placed in a one litre flask with screw cap and 400 ml of fresh maintenance medium was added The flask with a total volume of 800 ml was now placed on a magnetic stirrer in the 35 C incubator Samples of the cells were examined once a day in a phase contrast microscope for viral changes If necessary a proper amount of sodium bicarbonate was added to change the pH to neutral After incubation for 6-7 days the culture was harvested and

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centrifuged for 30 minutes at 20000 rpm in Spinco model L ultracentrifuge using rotor no 30 A 10 per cent suspension of sediment was prepared in 0 1 M glycine NaOH buffer pH 9 0 and incubated at 35 C for 8 hours with a vigorous shaking at hourly intervals The suspension was left overnight at 4 C and the antigen was sonicated with sonicator (MSE 100 watts model) for 5 minutes Finally the antigen was centrifuged in an International centrifuge (Model PR 6 Head 253) for 10 minutes at 2000 rpm The supernatant was the gel precipitation antigen and it was stored at +4 C The procedure for preparing control antigens was identical except the cells were not inoculated with rubella virus The potency of the control antigen was tested by gel precipitation against BHK 21 antiserum prepared in rabbits

HI and CF tests Disposable microtitre U plates were used The HI method is described by Halonen et al (4) In CF tests the microtechnique of Sever (12) was used Veronal buffered diluent (VBD) with 0 1 per cent gelatine was the diluent The haemolytic system consisted of equal parts of 2 per cent sheep erythrocytes and a dilution of haemolysin containing two full units Four antigen units and two full units of complement were employed in the test After overnight incubation at +4 C the haemolytic system was added and the plates were incubated at +37 C for 1 hour Then the plates were placed at +4 C for 1-2 hours and read by visual estimation of the degree of haemolysis The inhibition of haemolysis was recorded from 4+ to — and readings 4+ and 3+ were considered positive

Gel precipitation (GP) tests The micro modification of gel double diffusion described by Wadsworth (18) and modified by Kraus et al (6) was used The gels were prepared on the 5 cm × 5 cm photographic slide glasses The matrices with steel beads in their holes were put on the agar filmed slides Four nylon threads supported the matrix Agar was allowed to flow into the 0 4 mm high reaction chamber from one of the open sides After about 30 minutes the nylon threads were carefully removed and the sides of the matrix were sealed to the slide with agar Excess agar and the steel beads were removed from the holes by suction Special Agar Noble in phosphate buffered saline (PBS) pH 7 4 was used in a concentration of 1 per cent The gels also contained 0 01 per cent sodium azide Matrix holes were filled with 25 microlitres of reactant by sterilized disposable capillary pipettes After the reactants were added the slides were placed in humidified chambers The precipitations were allowed to form at room temperature The final reading was performed after 72 to 120 hours usually 84 hours In order to make the precipitation lines more visible the slides were washed and stained First the plexiglass matrices were carefully removed and the glass slides with agar sheet were washed in PBS for 24 hours PBS solution was changed 2–4 times The slides were then stained with Amido Black (2) without drying the agar Excess stain was washed away with 2 per cent acetic acid The slides were photographed on Agfa Agepan film and the results were read on the films with a magnifying glass

Plaque titration method The method is described by Vaheri et al (16)

The sera The sera from clinical rubella virus infections were sent to our laboratory from hospitals of Turku district and some of the sera were kindly supplied by Dr Leena Vihma (17) Random sampling of normal sera was carried out from the beginning of April to the end of August 1967 The children s specimens were from patients treated in the Children s Hospital of Turku University for diseases other than rubella The adult specimens were from the maternity clinic of the University Hospital The sera were treated with kaolin and chicken erythrocytes for HI tests (4) For CF tests the sera were inactivated at 56 C for 30 minutes In the gel precipitation tests untreated serum specimens were used

RESULTS

Demonstration of Precipitating Activity in Rubella Virus Antigens

Representative results of immunodiffusion reactions between a rubella antigen and a pair of sera from a rubella patient are shown in Fig 1 Serum no 1 was taken 2 months before rubella illness and serum no 2 about four months after the illness Rubella HI and CF titres were < 10 and < 4 in the first serum and 320 and 8 respectively

TABLE 1

Rubella HI and CF Titres and Occurrence of GP Antibodies in Sera from Rubella Patients

Patient	Weeks after the onset of disease	HI titre	CF titre	Number of GP lines
V M	0	<10	<4	0
	1	160	32	0
H O	0	160	4	0
	1	160	16	0
T A	0	<10	4	0
	1	40	4	0
S K	0	<10	4	0
	1	640	4	0
M H	0	160	<4	0
	2	≥640	32	0
V P	0	80	<4	0
	2	640	32	0
H R	0	20	<4	0
	2	40	32	0
T A	0	80	4	0
	2	≥640	8	1
P T	0	10	<4	0
	3	160	16	1
S P	0	<10	<4	0
	2	≥640	16	1
	3	640	16	1
	14	≥640	16	3
	21	320	16	2
T J	0	<10	<4	0
	18	640	8	1
T M	0	<10	<4	0
	18	≥640	16	1
M S	0	<10	4	0
	25	320	16	2
	38	≥640	16	2
K A	0	<10	<4	0
	5	≥640	16	1
	15	320	8	3
	23	80	16	2
K B	0	<10	<4	0
	3	160	16	2
	8	320	32	2
	61	160	8	1
M A C.	0	<10	<4	0
	4	320	32	1
	11	320	16	1
	63	160	8	1
I J	0	<10	<4	0
	3	320	32	0
	5	320	32	1
P A	0	<10	<4	0
	7	320	16	1
	11	320	16	2
	26	40	N D	1
R V	0	<10	<4	0
	6	320	32	1
	17	160	32	1

N D = not done

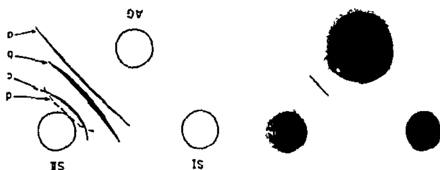


Fig 1

Immunodiffusion reactions between an alkaline extracted rubella antigen (AG) and a pair of serum specimens of a rubella patient The first serum (SI) was taken 2 months before rubella and the second serum (SII) 4 months after the illness Three clear precipitation lines are indicated by a b and c a faint line by d

in the second serum These sera exhibited no reactions with the BHK control antigens The first serum formed no precipitation line with the rubella antigen but the second serum formed at least three separate precipitation lines A fourth line (in Fig 1 line d) could be demonstrated in some experiments but it was very faint and only irregularly formed The lines a and b were easily reproducible but line c could not be found in some experiments In the ultracentrifugation of the antigen at 96000 g for 3 hours almost all GP activity remained in the supernatant only a very faint line (a) could be detected in the pellet but the whole HA activity was sedimented

TABLE 2

Relation of GP Reactions to the HI Titres in Randomly Selected Female Sera

HI titre	Number of GP positive sera			Number of GP negative sera	Total	% GP positive
	1 line	2 lines	3 lines			
<10	1	1	–	92	94	2
10	–	–	–	31	31	0
20	1	–		28	29	4
40	7	–	–	36	43	16
80	10	–	–	34	44	23
160	13	5	1	14	33	58
≧320	11	7		4	22	82
Total	43	13	1	39	296	19

The occurrence of GP antibodies in sera after rubella infections Th HI CF and GP results of some sera from clinical rubella infection are shown in Table 1 The HI antibody levels rise rapidly the CF titre more slowly and the HI and CF titres have a tendency to decrease dur

ing a longer observation period as demonstrated earlier (7 13) GP reactions were found in none of the sera during the first 10 days after the onset of illness but after two weeks 2 from 5 patients showed gel precipitation lines Almost all the sera taken 3 or more weeks after illness exhibited one or more precipitation lines The highest number of definite lines was three which were found 14 15 weeks after the onset of illness Later the number and intensity of lines showed a tendency to decrease but as long a time as 63 weeks after the illness there were still positive GP reactions (a line)

TABLE 3

Relation of GP Reactions to the CF Titres in Randomly Selected Female Sera

CF titre	Number of GP positive sera			Number of GP negative sera	Total	% GP positive
	1 line	2 lines	3 lines			
<4	–	1	–	91	92	1
4	4		–	34	38	12
8	9	1	–	13	23	43
16	8	2	1	8	19	58
≥32	3	3	–	2	8	75
Total	24	7	1	148	180	18

The occurrence of GP antibodies in randomly selected sera The GP reactions and HI titres of the randomly selected sera are indicated in Table 2 Two of the 94 HI negative sera demonstrated GP lines These sera gave however GP reactions with the control BHK antigen too Only one of the other 202 sera gave reactions with the control antigen This third serum was the only serum in Table 2 giving three GP lines One of these lines could be demonstrated with the control antigen Among the sera with low titres there were only a few showing GP antibodies to rubella About 25 per cent of sera with HI titres 80 had GP antibodies but more than 50 per cent of the sera with titre of 160 exhibited GP reactions The sera with HI titre 320 or more almost all had GP antibodies Except for one serum with titre < 10 the sera with titre 80 or lower had no more than one line All three sera having HI titres more than 640 had two GP lines About 20 per cent of all 296 sera examined had GP antibodies

In Table 3 are shown the results of the same sera correlated to CF titres Over 100 sera tested in HI were excluded because they showed anticomplementary activity More than 10 per cent of the sera having CF titres 4 showed GP reactions The sera with CF titres 8 had about 40 per cent positive GP reactions and if the titre was 32 or higher there were 75 per cent GP positive The distribution of GP reactions among the HI and CF positive sera in various age groups is shown in Table 4 It can be seen that the highest percentage of GP antibodies is

in the youngest age groups about 70 per cent The two oldest groups have only 10–20 per cent positive rate among rubella positive sera Only about 30 per cent of all rubella positive sera have GP antibodies when tested in this system

TABLE 4
Relation of GP Reactions to the Different Age Groups in Rubella HI and CF Positive Sera

Age group	HI positive sera: Number of GP positive sera	HI positive sera: Number of GP negative sera	HI positive sera: % GP positive	CF positive sera: Number of GP positive sera	CF positive sera: Number of CP negative sera	CF positive sera: % GP positive
6–8 years	8	4	67	8	3	73
10–12 years	14	6	70	10	3	77
17–22 years	18	45	29	7	19	27
23–29 years	12	52	19	4	18	18
30–35 years	3	40	7	2	14	13
Total	55	147	27	31	57	35

DISCUSSION

The results of the present study indicate that with the technique used reproducible precipitation reactions can be obtained with all aline extracted rubella antigens The specificity of the reactions was proved by acute and convalescent serum pairs of patients with serologically confirmed rubella and using a control BHK 21 antigen Only 3 of the 296 serum specimens tested showed reactions with the control antigens

Schmidt et al (11) were able to demonstrate immunodiffusion reactions with rubella virus using agarose and chamber technique but failed in initial attempts by using agar and slide technique However in the present study agar gel and a modified chamber technique produced at least three specific precipitation line The high sensitivity of this modified chamber technique has been demonstrated by *Krause et al* (6) by detecting concentrations as low as 0 03 microgram of antigen Later experiments in this laboratory have confirmed that agarose may be slightly better than agar A long incubation time for the development of the precipitation lines seemed to be necessary The first visible lines were observed after 36 hours but longer incubation times enhanced the line formation

The results of the serum specimens collected serially from rubella patients clearly demonstrate the late development of GP antibodies At the time of peak HI and CF titres GP antibodies were found only in a few serum specimens The heaviest lines and the highest number of lines were demonstrated as late as 4 months after the illness A similar late appearance of GP antibodies has been observed with the respi

ratory syncytial virus and arboviruses in immunized animals (1 5) and in experimental herpes keratoconjunctivitis (8) In this connection it is also interesting to note the very late occurrence of neutralizing antibodies to rubella virus demonstrated by *Leerhoy* (7) In the randomly selected sera the presence of GP antibodies was in good correlation with the results of HI and CF tests Practically no serum with low HI and CF titres showed GP reactions and almost all with high HI and CF titres had positive GP reactions There was also some correlation between the HI and CF titres and the number of the GP lines which increased in number when HI and CF titres were higher In the HI and CF positive sera the rate of GP positive specimens decreased in older age groups This may be correlated with the significantly higher number of higher HI and CF titres in younger age groups than in the older (9)

The antigens used in the present study were soluble They remained in the supernatant when all the haemagglutinating activity was pelleted It means that the viral particles and infectivity (14) were already at the bottom A faint activity was also demonstrated in the pellet as has been reported by *Schmidt et al* (11) The most constantly demonstrated line was the a line It was clearer and heavier than the other lines and because it was nearest to the antigen well it may be due to an antigen of larger size than the others The b line was the second from the antigen well and the c line the third The specificity of the irregular and faint d line requires further studies

An important application of the developed GP test is in the identification of rubella virus soluble antigens For the routine diagnostic work the developed test may not be practical However the very late appearance of GP antibody may make the gel precipitation test applicable for serological diagnosis of rubella in cases where the first specimen has been obtained several weeks after the onset of illness

SUMMARY

A gel diffusion (GP) technique using an alkaline extracted antigen was developed for rubella virus At least 3 virus specific precipitation lines were demonstrated The precipitating antigens were soluble only traces of these were sedimented by ultracentrifugation

The precipitating antibodies appeared two weeks or later after the onset of rash The highest number of GP lines were seen 4 months after the illness Among randomly selected sera only 20 per cent had precipitating antibodies The occurrence of GP antibodies was in correlation with the HI and CF titres More than 50 per cent of the persons having HI titre of 160 or more and CF titre of 16 or more had GP antibodies The highest percentage of GP antibodies was found in the youngest age groups

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COMBINED USE OF FLUORESCENT ANTIBODY TECHNIQUE AND CULTURE ON SELECTIVE MEDIUM FOR THE IDENTIFICATION OF *NEISSERIA GONORRHOEAE*

By

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Received 9 xii 68

In a previous study on identification of *Neisseria gonorrhoeae* (*N gonorrhoeae*) a comparison was made of the results obtained by culture and by fluorescent antibody test (FAT) (3) When FAT was performed after enrichment of the material by culturing (the delayed FAT) it was found to be slightly more sensitive than conventional culture followed by bacteriological identification The higher yield of positive results originated from the more heavily contaminated specimens especially from rectal swabs

During recent years a selective medium for isolation of gonococci was gradually developed and finally established by *Thayer & Martin* in 1964 (9) They combined the addition of ristocetin recommended by *Berger* in 1961 (1) with the addition of polymyxin B sulphate and nystatin This medium called TM medium (after *Thayer & Martin*) was found to give results superior to those obtained by *Deacon*s delayed FAT (2) when incubation on the TM medium was carried out for 40 hours (6)

Since 1965 the Neisseria Department at Statens Seruminstitut has used a selective medium of the TM type for routine identification of *N gonorrhoeae* (11) In the present experiments the results of culture on this medium were compared with those obtained by delayed FAT after inoculation on to both selective and non selective medium

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The clinical specimens were kindly supplied by the doctors at the Venereological Out Patients Department at Rudolph Berghs Hospital Copenhagen (Head *A Perdrup* MD PhD)

This study was supported by grants from the *World Health Organization*

MATERIALS AND METHODS

Media

Selective medium The HYL medium described by *Møller & Reyn* (7) was used but with one modification *i e* haemoglobin was replaced by defibrinated horse blood (12) The following antibiotics were added polymyxin B sulphate (Pfizer) 25 IU Mycostatin® (Squibb) 25 IU and Spontin® (Abbott) 10 µg/ml

Non selective medium The same medium without antibiotics

Fluorescent Antibody Test (FAT)

Preparation of rabbit antigonococcal sera and rabbit antistaphylococcal sera labelling with fluorescein isothiocyanate (FITC) and performance of the test were as described previously (3 4)

Reading ++++ denotes a brilliant yellowish green fluorescent layer covering uniformly the surface of each bacterial cell + ++ and +++ indicate increasing degrees of homogeneity and intensity of the fluorescence of a bacterial population 0 denotes a pale bluish fluorescence (barrier filter Zeiss No 47)

The specific positive reaction is characterized by the morphology of the microorganisms *in casu* typical diplococci and the degree of fluorescence (+++ and ++++)

Bacterial Strains (see Table 1)

Treatment of Specimens

Duplicate specimens were obtained from patients attending the Venereological Out Patients Department at Rudolph Berghs Hospital Copenhagen The specimens were transported to the laboratory on charcoal impregnated sterile wooden applicators in a modified Stuart medium (10) The transportation time never exceeded six hours

One swab was used for identification of gonococci by conventional culture on selective medium The gonococci were identified by Gram staining oxidase reaction and fermentation tests

The other swab was inoculated on to both selective and non selective medium the two media being used alternately After 18 hours incubation at 36 C in a moist atmosphere containing 10 per cent carbon dioxide duplicate smears were made from each plate irrespective of whether growth was visible or not The slides were fixed in methanol and stained with a mixture of FITC labelled rabbit antigonococcal globulin and unlabelled rabbit antistaphylococcal serum (3 4) The stained smears were examined by fluorescence microscopy on the same day The corresponding plates were kept until the results obtained by conventional culture were available When culture was negative and delayed FAT positive the identity of the strain was confirmed by the usual bacteriological tests

In the case of specimens received on Saturday afternoons the procedure was slightly modified *i e* the swab intended for routine culture was inoculated immediately but the one for FAT was kept in Stuart medium at 4 C until Monday morning and then used as described above

RESULTS

Preliminary and Supplementary Experiments

Fifty freshly isolated strains of *N gonorrhoeae* were examined by FAT after growth on both selective and non selective medium The antibiotics did not induce changes in morphology nor did they alter the stain ability of the gonococci

Various strains of saprophytic *Neisseria Mima* species *Herellea* species and *Moraxella* species plus 14 strains of *Staphylococcus aureus* were inoculated on to both selective and non selective medium On selective medium growth of all strains except some *N catarrhalis* and

TABLE 1

Growth of Various Strains on Selective and Non Selective Medium
FAT Performed on Smears from Selective Medium

Strains		Growth on		FAT	
		Selective medium	Non selective medium	I	II
N. catarrhalis	ATCC 8193	1	4	++	0
N. catarrhalis	ATCC 8176	0	4	++	0
N. catarrhalis	ATCC 7900	2	4	0	0
N. catarrhalis	NCTC 4103	1	4	+	0
N. catarrhalis	SS 465/2	4	4	++	0
N. catarrhalis	SS 8/895	4	4	++	0
N. flava	ATCC 14221	1	4	+	0
N. perflava	ATCC 10555	1	4	0	0
N. subflava	ATCC 11076	0	4	0	0
N. flavescens	ATCC 13170	1	4	++	0
N. sicca	ATCC 9913	0	4	+	0
N. haemolysans	ATCC 10349	0	4	0	0
N. caviculi	ATCC 14684	0	4	0	0
N. cuniculi var gigant	ATCC 14689	1	4	0	0
N. canis	ATCC 14687	2	4	0	0
N. caviae	ATCC 14659	0	4	0	0
N. denitrificans	ATCC 14686	0	4	+	0
Mima polymorpha	ATCC 9957	2	4	+	0
Mima polymorpha	ATCC 10973	3	4	0	0
Herellea species	ATCC 9955	2	4	+	0
Herellea species	ATCC 9954	2	4	0	0
Herellea species	ATCC 11959	2	4	0	0
Moraxella bovis	ATCC 10900	0	4	0	0
Moraxella species	ATCC 12499	1	4	0	0
S. aureus (7 reactive strains)		1	4	++++	0
S. aureus (7 non reactive strains)		1	4	0	0
N. gonorrhoeae (URI)	ATCC 11688	4	4	++++	++++
N. gonorrhoeae (J G)	ATCC 11689	4	4	++++	++++
N. gonorrhoeae (fresh control)		4	4	++++	++++

I Stainability with FITC-labelled rabbit antigonococcal globulin
II Stainability in one step inhibition test i e with a mixture of FITC labelled rabbit antigonococcal globulin and unlabelled rabbit antistaphylococcal serum cf reference No 4

one strain of *Mima polymorpha* var *oxydans* was strongly or completely inhibited (Table 1) The degree of inhibition was estimated roughly by comparison with the growth on non selective medium Macroscopically identical results were recorded as 4 and 25 per cent 50 per cent 75 per cent and 100 per cent inhibition as 3 2 1 and 0 respectively In order to obtain more quantitative information concerning the degree of inhibition a few representative strains were retested using a known inoculum followed by colony counting For instance on selective medium *Staphylococcus aureus* (growth degree 1 on selective medium) produced < 10 colonies from an inoculum of 2×10^7 cells *Mima polymorpha* ATCC 9957 (growth degree 2 on selective medium) produced < 10 colonies from 10^7 cells and *Mima polymorpha* ATCC

10793 (growth degree 3 on selective medium) produced 150 colonies from 10^4 cells. Even *N. catarrhalis* 160/2 (growth degree 4 on selective medium) was inhibited thirty fold.

As regards all strains listed in Table 1, duplicate smears were made from the selective medium whether or not there was visible growth. One smear was stained with antigonococcal conjugate diluted in saline and the other with the usual mixture of antigonococcal conjugate and unlabelled rabbit serum. The antibiotics used in the selective medium did not induce any changes in stainability as compared to previous examinations (3). The two gonococcal strains ATCC 11688 and 11689 were included because they had reacted poorly with antigonococcal conjugate in a previous experiment (3). They now showed a typical specific reaction.

Lucas et al. (5) reported that after exposure to lethal doses of penicillin, streptococci of groups A and B changed their stainability with antigonococcal conjugate. Three streptococcal strains of groups A, B and G were examined by FAT before and after treatment with 1000 units of penicillin per ml for 24 hours without the stainability of the strains being altered. The one group A strain, which was previously shown to react non-specifically (3), was stained both before and after treatment with penicillin and this staining was inhibited when unlabelled rabbit serum was added to the conjugate.

Finally, it was examined whether the stainability of gonococci was influenced by testing with oxidase reagent (tetramethyl-p-phenylene diamine hydrochloride). Gonococcal colonies found after 24 and 48 hours incubation of primary cultures and 18 hour subcultures were examined at various times after the addition of oxidase reagent (from half an hour to 24 hours). Simultaneously a subculture was made as a test of viability. The oxidase reagent neither induced changes in stainability nor killed the bacteria.

Identification of N. gonorrhoeae by Means of FAT after Enrichment of the Material on Selective and Non-Selective Medium

Table 2 shows the results obtained by culture on selective medium followed either by bacteriological or immunofluorescent identification of the gonococci. In addition, the FAT was performed after inoculation on to non-selective medium. The material consisted of 815 duplicate specimens obtained from patients attending a venereological clinic; the patients were either suspected of suffering from gonorrhoea or they were under control for the effect of treatment. The results obtained by FAT from swabs which had been kept from Saturday to Monday in the icebox did not differ from the rest of the material and were therefore included without comment.

The agreement between results obtained by conventional culture and by FAT was good. By both methods 610 specimens were negative and

TABLE 2

Identification of N gonorrhoeae in 815 Duplicate Specimens
Comparison of Culture and Delayed FAT

Culture on selective medium		FAT positive			FAT negative	Total
		Both media	Selective medium	Non selective medium	Both media	
Positive	Day 1	131	7	1	3	142
	Day 2	44	5	1	5	55
	Total	175	12	2	8	197
Negative		4	2	2	610	618
Total		179	14	4		
		197			618	815

Day 1 = visible colonies after 18–24 hours incubation
Day 2 = visible colonies after 48 hours incubation

TABLE 3

Identification of N gonorrhoeae in 175 Female Patients (456 Duplicate Specimens)

	Culture	FAT positive				FAT negative	Total
		Both media	Selective medium	Non selective medium	Total	Both media	
Patients	positive	50	2	0	52	2	54
	negative	0	0	1	1	120	121
	total	50	2	1	53	122	175
Urethra	positive	37	7	1	45	1	46
	negative	2	0	1	3	126	129
	total	39	7	2	48	127	175
Cervix	positive	45	0	0	45	4	49
	negative	0	1	0	1	119	120
	total	45	1	0	46	123	169
Rectum	positive	12	3	0	15	3	18
	negative	1	0	0	1	93	94
	total	13	3	0	16	96	112
All sites	positive	94	10	1	105	8	113
	negative	3	1	1	5	338	343
	total	97	11	2	110	346	456

189 were positive The remaining 16 specimens were distributed as follows 8 positive by culture alone and 8 by FAT alone

By culture on selective medium 197 specimens were found positive 70 per cent of which had shown visible gonococcal colonies after 24

hours incubation The remaining 30 per cent were detected after 48 hours specimens received on Saturdays afternoons included

Only one of the gonococcal strains was unable to grow on the selective medium but was detected by FAT on smears from non selective medium The strain was identified by the usual bacteriological tests and the inhibition of growth on selective medium was confirmed

As regards specimens from male patients the discrepancies in the results obtained by the different methods were negligible The results of 156 duplicate specimens from female patients are shown in Table 3 Only 13 specimens gave differing results 8 were found positive by culture alone and 5 by FAT alone The most striking figures are firstly the high degree of agreement between the results of culture and FAT in the case of rectal specimens and secondly that four cervical specimens were positive by culture and negative by FAT In all four cases very few (1–5) gonococcal colonies were found after 18 hours incubation

TABLE 4

Identification of N gonorrhoeae by FAT (1630 Duplicate Smears)

	Culture on	Both smears positive	One smear positive	Both smears negative
Males	Selective medium	85	1	973
	Non selective medium	80	5	974
Females	Selective medium	101	10	345
	Non selective medium	96	4	356
Total		362	20	1948

TABLE 5

Identification of N gonorrhoeae Comparison of FAT and Culture

FAT	1st series Culture on non selective medium 674 specimens		2nd series Culture on selective medium 815 specimens	
	Positive	Negative	Positive	Negative
Positive	173	32	189	8
Negative	5	454	8	610

Table 4 illustrates the significance of making duplicate smears from each plate for the FAT In specimens from male patients the advantage is minimal when selective medium is used for enrichment However under the same conditions 9 per cent (10/111) of the gonococci found in specimens from patients were detected in only one of the duplicate smears

Table 5 shows a comparison of results obtained by FAT and culture before and after introduction of the selective medium into our routine culture method In the first series when non selective medium was used both for bacteriological identification and for enrichment in FAT the advantage of the FAT was significant a further 13 per cent (27/210) positive results were found and these were available much earlier In the second series after the introduction of selective medium, the two methods detected the same percentage of positive results However the results of FAT were available 24–48 hours earlier than those obtained by culture

DISCUSSION

Since Deacon in 1959 introduced the use of Coons immunofluorescent technique in the diagnosis of gonorrhoea the reliability and sensitivity of this test have been confirmed by several workers In order to get results equal to those obtained by conventional culture an interposed enrichment of the material by inoculation was found necessary This fluorescent antibody test (delayed FAT) was often found to be more sensitive than culture followed by bacteriological identification even when the latter was performed under optimal conditions The selective TM medium introduced by *Thayer & Martin* in 1964 was claimed to give results superior to *Deacon*'s delayed FAT when incubation on this medium was carried out for 40 hours (6) These recent developments in the laboratory diagnosis of gonorrhoea have been reviewed by *Reyn* (11) in a paper which includes a comprehensive list of references

The present experiments were performed firstly to evaluate the use of selective medium for enrichment of material to immunofluorescent identification of *N gonorrhoeae* and secondly to examine how often gonococci were inhibited by the antibiotics in a selective medium of the TM type It was demonstrated in preliminary experiments that the antibiotics used in the selective medium neither induced morphological changes in gonococci nor changed the specific stainability Furthermore the antibiotics did not induce non specific reactions of the other bacteria tested (Table 1) and these were also found to be so strongly inhibited that they only occasionally grew out after inoculation of clinical specimens The addition of oxidase reagent (tetramethyl p phenylene diaminehydrochloride) to primary cultures of gonococci neither altered the specific staining reaction nor killed the bacteria Thus subsequent subculturing for sensitivity determinations was not biased The latter result is not in accordance with the observations by *Peacock et al* (8) presumably because these authors used the more damaging dimethyl p phenylene diaminehydrochloride as reagent

The results obtained in the case of 815 duplicate specimens from patients suspected to be suffering from gonorrhoea or under control following treatment confirmed the usefulness of both methods Compari

son with a previous examination of specimens from a similar group of patients attending the same clinic (3) (Table 5) proved that the introduction of selective medium had eliminated one of the advantages of FAT viz. the higher yield of positive results obtained from heavily contaminated specimens

It should be mentioned that FAT has been given some handicaps in these experimental series The swab used for bacteriological identification was streaked on to one plate while the swab intended for FAT was used twice Furthermore the inoculum was spread as in bacteriological procedures (10) This implies a dilution of the FAT specimen which may be particularly critical when a selective medium is used for enrichment over an 18 hour period (prolonged generation time decreased germinating power) This is illustrated by the unexpected finding that four cervical specimens were positive by culture and negative by FAT only one positive result was found by FAT alone (Table 3) Earlier experiments had proved the relationship to be the opposite The same problem is reflected in the finding that about 10 per cent of the gonococci from female patients were detected in only one of the duplicate smears thus indicating that the number of organisms present after 18 hours was still very small

The statement by Lucas et al (5) that FAT "is unsuitable for testing the efficacy of penicillin and other antibiotic therapy since residual dead bacterial forms or cross reacting organisms may be present in the specimens could not be confirmed Neither could it be confirmed that penicillin induces non specific staining in group A and B streptococci at least not in the few strains tested by the author

Only one strain was encountered which did not grow on the selective medium The inhibitory agent was ristocetin 10 µg/ml (11) The gonococcal strains isolated in Denmark today are generally more sensitive to the antibiotics commonly used for treatment than was the case a few years ago Since strains sensitive to less than 10 µg ristocetin tend to occur mainly among strains which are sensitive to penicillin the proportion lost on selective medium may now be higher than at the time when the experiments reported were performed Ristocetin has recently been replaced by vancomycin (3 µg/ml) in our routine medium Although vancomycin seems to be less inhibitory to gonococci than ristocetin recent investigations by Reyn (11) indicate that about 3 per cent of the present population of gonococcal strains are sensitive to 3 µg vancomycin/ml

It may be concluded that ideally each specimen should be inoculated both on to selective medium for bacteriological identification and on to non selective medium for FAT the latter possibly being performed after localization of suspicious colonies by means of the oxidase reaction It would thus be possible both to detect a very small number of gonococci in a specimen and to detect strains that do not grow on the selective medium

SUMMARY

A series of 815 duplicate specimens was obtained from patients attending a venereological out patients department N gonorrhoeae was identified by culture on selective medium of the Thayer Martin type and by fluorescent antibody test after enrichment of the material by culturing on both selective and non selective medium The total number of positive results was 197 Out of these 179 were positive by both methods 8 by culture alone and 8 by fluorescent antibody test alone One strain was encountered which was not able to grow on the selective medium i e it was inhibited by ristocetin (10 μg/ml)

Thus the percentage of positive results found by inoculation on to selective medium was the same whether identified bacteriologically or by the fluorescent antibody test However the results obtained by the immunofluorescent technique were available 24 48 hours earlier than those obtained from the routine procedure

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ANTIBODY INDUCED SUPPRESSION OF THE IMMUNE RESPONSE IN ANTIGEN STIMULATED CULTURES

By

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Received 23 xii 68

In the intact animal inhibition of the cellular and humoral immune response has been observed when specific antibodies are administered together with the stimulating antigen (*Uhr & Baumann* 1961 *Moller & Wigzell* 1965 *Wigzell* 1966) Humoral antibodies have been regarded as feed back factors regulating the cellular immune response (*Uhr & Moller* 1968) *Moller & Wigzell* (1965) and *Wigzell* (1966) have done extensive studies on the effect of γM and γG antibodies on the humoral response and the formation of cells with plaque forming ability in spleen suspensions from mice immunized against sheep red cells They presented evidence that antibody exerts an inhibitory effect on the antigen whether it is extracellular or in phagocytic cells in the spleen but found no evidence that passively administered antibodies had any direct inhibitory effect on antibody synthesizing cells

The purpose of the present work was to study the effect of antibodies on an antigen induced immunological reaction *in vitro* The effect of anti sheep red cell antibodies on cellular proliferation and specific immune response in sheep red cell stimulated cultures containing blood lymphocytes from sheep red cell immunized rabbits was investigated

MATERIAL AND METHODS

Cell Cultures

The cell cultures were prepared with lymphocytes from sheep red cell (SRC) immunized rabbits as previously described (*Lamvik* 1968 a) The cultures were stimulated with SRC as outlined below They were harvested after 8 days culturing apart from two culture series that were harvested after 6 days The culture medium was changed after 4 days incubation The culture medium was prepared with 4 parts of Parker s tissue culture medium (TC199) and 1 part of rabbit serum Each culture tube contained 2 5 × 10^6 cells suspended in 2 5 ml of medium One half ml of TC199 with 1 per cent SRC was added to each tube as stimulator while 0 5 ml of TC199 without SRC was added to each of the control tubes

In one series of cultures rabbit serum with varying amounts of complement was used in the medium Two pooled rabbit serum samples one fresh containing com

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plement the other inactivated at 56° C for 30 min were mixed in different proportions to give a relative variation in fresh serum content in the final medium from 0 to 20 per cent

Anti SRC Antibody Sera

Two sera were tested for suppressive effect on the cellular reactions

a) One rabbit was bled 5 days after one single intravenous injection of 1 ml of 5 per cent washed SRC The serum which is called early serum showed an agglutinin titre of 64 and a lytic titre of 1024

b) One other rabbit was bled 4 weeks after the end of a 6 week immunizing course consisting of one intravenous injection of 1 ml and 14 injections of 0 5 ml of 35 per cent washed SRC The serum which is called late serum" showed an agglutinin titre of 512 and a lytic titre of 1024

The early and late sera were both diluted 1/2 1/20 and 1/200 in pooled normal rabbit serum which had been adsorbed with SRC to remove heterophil antibody (*Kabat & Mayer* 1961) The immune serum dilutions were then used in the culture experiments in two ways

In most experiments the SRC used for stimulating the cultures were pre incubated in inactivated immune serum dilutions (1 per cent cell suspension) for 1 hour at room temperature washed three times in phosphate buffered saline and suspended in TC199 to 1 per cent concentration In this way antibodies were adsorbed to the antigen in varying amounts before the antigen was added as stimulator to the culture tubes Control cultures were stimulated with SRC pre incubated in inactivated normal rabbit serum or with non incubated SRC or were cultured without stimulation Pooled adsorbed rabbit serum was used in the culture medium at the start of culturing as well as for the change of medium

In some culture experiments the immune serum dilutions were used in the culture media as the only serum addition These cultures were stimulated with normal SRC suspended in TC199 Control cultures were prepared with adsorbed and non adsorbed normal rabbit serum with and without sheep red cell stimulation At the time of medium change pooled adsorbed rabbit serum was added to all culture tubes

Cell Reaction

The cell reaction in the cultures was measured at the time of harvesting by testing for plaque forming ability in SRC monolayers in micro incubation chambers as previously described (*Iamoil* 1968 b) The total cell numbers and differential counts in the incubation chambers were determined after the addition of acridine orange stain The numbers of plaque forming cells per 10^4 harvested cells were estimated The agglutinin titres in the culture supernatants after 4 days incubation (medium change) and at the time of harvesting were also determined

RESULTS

No definite effect of complement content on the blastoid transformation was observed in culture tubes containing varying relative amounts of fresh rabbit serum in the culture medium (*Table 1*) A slight but insignificant increase in the numbers of plaque forming cells was noticed concomitant with the increase in complement content However cultures without complement in the medium and without visual lysis of the erythrocytes added as stimulator still showed blastoid transformation with development of plaque forming ability Before testing cells cultured without complement in the medium for plaque forming ability in micro incubation chambers any intact SRC still present in the culture tubes had to be eliminated by immune lysis at 37 C for

	Cultured cells	Medium	Stimulator
a	Blood lymphocytes from SRC immunized rabbits	TC 199 with normal rabbit serum	SRC incubated in immune serum diluted 1/2 1/20 1/200
b	Blood lymphocytes from SRC immunized rabbits	TC 199 with immune serum diluted 1/2 1/20 1/200	 SRC SRC SRC

Fig 1

Experimental programme for testing the effect of antibodies on the immune response *in vitro* The culture variants were checked at the time of harvesting for blastoid transformation plaque forming cells and agglutinins Control culture variants are described in the text

30 min after addition of complement Without this procedure the unlysed SRC which were coated by anti SRC antibodies present in the culture medium were transferred together with the lymphocytes to the incubation chambers and gave diffuse lysis in the sheep red cell monolayers

TABLE 1

Cellular Transformation and Numbers of Plaque Forming Cells in Lymphocyte Cultures Containing Varying Amounts of Complement

Fresh serum content of medium relative amounts %	Cellular transformation		Plaque forming cells per 10^{4} harvested cells
	All transformed cells %	Blastoid cell	
20	44 75±[illegible]	9 0 ±[illegible] 0	43 8± 8 5
10	4[illegible] 25±1 75	7 7 ±0 [illegible]5	36 3±13 0
[illegible]	44 0 ±1 5	[illegible] 75±0 75	[illegible]3 3± 9 1
2	38 0 ±3 5	8 5 ±0 5	[illegible]4 3±11
0	37 75±4 [illegible]5	7 [illegible]5±1 [illegible]	24 3±11 [illegible]

Means and range of results from duplicate culture tubes

In cultures stimulated with SRC pre incubated with different amounts of early and late immune serum (Fig 1a) no suppressive effect was observed on the blastoid transformation A suppressive effect on the specific immune response was however found Fig 2 shows the results of one of several similar culture series

Late immune serum in high concentration appears to counteract

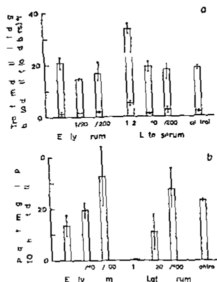


Fig 9

Cellular transformation (a) and numbers of plaque forming cells (b) in cultures containing lymphocytes from SRC immunized rabbit *In vitro* stimulator SRC pre incubated in early or late immune serum diluted 1/2 1/20 and 1/200 in normal adsorbed rabbit serum Control cultures stimulated with SRC without pre incubation Means and range of results from duplicate culture tubes after 8 days culturing

the stimulating action of SRC so that no plaque forming cells develop in the cultures Late immune serum diluted 1/20 gave a moderate reduction in the numbers of plaque forming cells compared to the effect of immune serum diluted 1/200 Early immune serum in different concentrations showed a similar effect on the stimulating ability of SRC The differences between the results following the use of different serum dilutions were however slight and probably not significant Cultures stimulated with SPC pre treated with high dilutions of immune sera gave about the same numbers of plaqueforming cells as cultures stimulated with non treated SRC

The pooled results from two other culture series are shown in Fig 3 Late immune serum was used for preincubating the SRC before addition to the culture tubes The results are compared with the results in cultures stimulated with SRC pre incubated with normal rabbit serum containing trace amounts of anti SRC antibodies Again a definite suppressive effect of late immune serum on the stimulating action of SRC on the development of plaque forming cells was found (Fig 3a) Serum diluted 1/20 had an effect intermediate between that of normal serum and immune serum diluted 1/2 The same trend was observed with the agglutinin titres in the culture supernatants (Fig 3b) In the culture tubes stimulated with SRC pre incubated with low dilutions of immune serum agglutinins in low titres were found at the time of

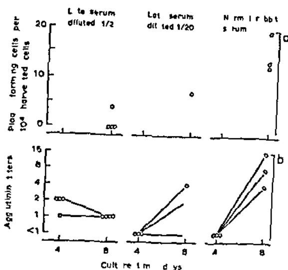


Fig 3

Numbers of plaque forming cells at harvesting and agglutinin titres at time of medium change and at harvesting in lymphocyte cultures from two SRC immunized rabbits *In vitro* stimulator SRC pre incubated in immune serum dilutions and in normal rabbit serum as indicated Eight days culturing

medium change probably due to liberation of antibodies from the SRC No increase in agglutinin titres was noticed during the last 4 days of culturing In cultures stimulated with SRC pre treated with immune serum in higher dilutions or in normal rabbit serum agglutinins ap peared in the supernatants in the last days of culturing The agglu tinin titres were highest in the latter culture variant

In culture series stimulated with SRC and containing varying amounts of late immune serum in the culture medium (Fig 1b) re sults similar to those outlined above were obtained Immune antibodies caused suppression of the development of plaque forming cells while no definite effect was observed on the degree of cellular transformation in cultures harvested on the 6th (Fig 4) as well as on the 8th day of culturing The suppressive effect on the specific immune response was usually stronger than in culture variants stimulated with SRC pre incubated in immune serum dilutions possibly due to higher antibody content in the former cultures

The supernatants in the culture variants to which immune serum in high concentrations had been added at the culture start contained agglutinins at the time of medium change also These passively added agglutinins were still present in small amounts in the supernatants after change of medium since the cells were not washed at the time of medium change Due to this contamination any slight new synthesis of agglutinins would not be detected in cultures prepared with im mune serum diluted 1/2 and 1/20 In cultures prepared with immune serum in high dilution and with normal SRC *adsorbed* or non

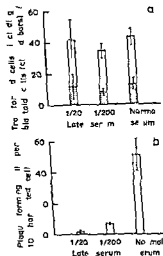


Fig 4

Cellular transformation (a) and numbers of plaque forming cells (b) in SRC stimulated cultures containing different dilutions of late immune serum in the medium compared to cultures containing normal rabbit serum Means and range of results from duplicate culture tubes after 6 days culturing

adsorbed rabbit serum agglutinins appeared between medium change and harvesting on the 8th day

DISCUSSION

The first part of this study was concerned with the possible effect of complement on the stimulating effect of SRC in blood lymphocyte cultures Culture series containing varying amounts of fresh serum demonstrated no definite effect of complement on cell transformation and development of plaque forming cells Another observation is probably related to these findings Culture medium containing SRC adsorbed serum supported the cellular reactions against SRC as did medium containing non adsorbed serum although the strongest reactions were usually found in cultures with the small amount of anti SRC lytic antibodies (heterophil antibodies) present in the pooled normal rabbit serum used in the medium with non adsorbed serum Thus the stimulating effect of SRC on the cultured primed lymphocytes is not dependent on though it may be facilitated by lysis of the added SRC

The main experiments outlined in Fig 1 concerned the possible suppressive effect of immune antibodies on the stimulating effect of SRC Late immune serum with strong SRC agglutinating ability was found to suppress the development of plaque forming cells completely and at the same time to prevent any detectable liberation of anti SRC agglutinins This latter effect could only be evaluated when immune

serum was used for pre incubation The suppressive effect was found to depend on the amount of immune antiserum used Although the estimation of plaque forming cell numbers in *micro incubation chambers gives only semi-quantitative data* on the immune response (*Lamvik* 1968b) the same trend was observed in all culture series

Early immune serum with high lytic and low agglutinating ability towards SRC showed in low dilutions only a weak and insignificant *suppressive effect on the development* of plaque forming cells

Late immune serum added directly to the culture medium gave the same effect as *pre incubation of the SRC* in the immune serum dilutions The antibodies added to the media would presumably be bound rapidly to the *stimulating SRC so that the final effect of the antibodies* in the cultures would be the same The suppressive effect was usually more complete *when immune serum was added to the medium* possibly due to larger amounts of antibodies applied in this way

No suppression of cellular transformation was *seen in the culture* tubes to which immune antibodies had been added despite the signs of *inhibition of the immune response* which *were demonstrated* The morphological evaluation of cellular transformation was done in vital preparations stained by acridine orange The harvested cells were well separated in such preparations Cell differentiation was performed without difficulties due to clear nuclear and cytoplasmatic staining in all intact cells Permanent preparations fixed and stained with May Grunwald Giemsa demonstrated also clearly the cell transformation in the cultures where the specific immune reaction had been suppressed by addition of immune serum Due to clumping of the cells accurate differential counts could not be done in such preparations however

The results show that inhibition of the complete immune response with suppression of antibody synthesis may occur without inhibition of the cell proliferation after antigen stimulation Cell proliferation normally precedes the development of antibody synthesizing cells *in vitro* as well as *in vivo* (*Baney Vazquez & Dixon 1961*) *Moller & Wigzell* (1965) found that humoral antibodies given to mice a few days after the immunizing antigen gave suppression of antibody liberation after a latency period This observation indicates that antibodies do not inhibit antibody synthesis in already committed cells They *thought* that the principal *in vivo* effect of the addad antibodies was to remove or counteract the stimulus for proliferation of the precursors of antibody producing cells This view is supported by the findings by *Rowley & Fitch* (1964) and *Sahiar & Schwartz* (1966) that the increase in spleen weight and the morphological changes which accompany the primary response *in vivo* is suppressed by antibodies given together with the antigen *Rowley & Fitch* found no suppressive effect of antibodies on the secondary response *in vivo* The findings in the present *in vitro* culture experiments which may be comparable to the secondary

in vivo response, suggest an inhibitory effect of the added antibodies on the specific gammaglobulin synthesis which probably occurs in the progeny of the proliferating cells without suppression of antigen induced cell proliferation. The differences between the *in vitro* results and the previously reported *in vivo* findings may possibly be explained by quantitative differences in stimulating or suppressing agents. The importance of the quantity of the stimulating agent is suggested by the findings by *Rowley & Fitch* (1964) that small doses of antigen induced unresponsiveness to subsequent injections of larger doses of the antigen in contrast to the usual priming effect of larger antigen doses. Antigen antibody complexes have been reported to give an increased antibody response in some *in vivo* experiments (*Uhr & Möller* 1968).

Our *in vitro* results may be explained by a change in the specificity for the induction of cell proliferation. When antigen is added to the primed lymphocytes cell proliferation occurs followed by development of antibody producing cells. If antigen antibody complexes are added as in the antibody inhibition experiments the complexes may possibly exert a non specific stimulating action on the lymphocytes similar to the well known non specific blastogenic agents like bean extracts and bacterial filtrates. A non specific blastogenic effect of antigen antibody complexes on human blood lymphocytes has recently been reported by *Bloch Shtacher, Hirschhorn & Uhr* (1967). The mechanism of specific and non specific induction of blastoid transformation is as yet unknown. However the present results indicate that antibodies may suppress the specific immune reaction of lymphocytes which nevertheless are able to proliferate following different types of stimulation.

SUMMARY

Serum from sheep red cell immunized rabbits caused an inhibition of the immune response in cultures containing blood lymphocytes from sheep red cell immunized rabbits that were stimulated with sheep red cells. A suppression of the development of plaque forming cells and the liberation of agglutinins was observed. No inhibition of cellular blastoid transformation was however observed. Antigen antibody complexes seem to give rise to a non specific cellular reaction in the cultures without the immune response which follows when pure antigen is used as stimulator.

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ANTIBODY SYNTHESIS IN CULTURES OF BLOOD LYMPHOCYTES FROM TYPHOID IMMUNIZED RABBITS FOLLOWING STIMULATION IN VITRO WITH KILLED TYPHOID BACTERIA

By

J O LAMVIK

Received 23 xii 68

Previous experiments (*Lamvik* 1968a 1969c) have shown that blood lymphocytes from sheep red cell (SRC) immunized rabbits were able to transform from a resting stage to specific antibody synthesis This transformation is dependent on antigen stimulation *in vitro* Antibody production was demonstrated in two ways by the presence of plaque forming cells with lytic action towards SRC and of anti SRC agglutinins in the culture medium

This report deals with the use of bacterial antigen as stimulator in cultures containing blood lymphocytes from rabbits immunized with the same bacterial antigen Fig 1 shows the experimental programme in general terms including the tests performed for signs of antibody production Gram negative entero bacteria are convenient for immunization and stimulation since they contain lipopolysaccharides easily coated on erythrocytes (*Landy, Trapani & Clark* 1955) which then may be used for testing the cultured cells for plaque forming ability and the culture supernatants for liberated agglutinins

MATERIAL AND METHODS

Antigens

Salmonella typhosa killed by heat at 60 C for 1 hour was used for *in vivo* immunization and *in vitro* stimulation Lipopolysaccharide from *S typhosa* was obtained from Difco Detroit Mich The lipopolysaccharide (code 3946) prepared by Boivin extraction was coated to SRC by one of the methods described by *Landy Trapani & Clark* (1955) The material was treated with alkali (0 02N NaOH in saline) at 37 C for 12 h Following neutralization with HCl a solution containing 10 µg of lipopolysaccharide per ml was prepared Washed SRC were made up to a 1 per cent suspension in saline mixed in equal volumes with the lipopolysaccharide solution and incubated at 37 C for 2 hours followed by washing three times in saline

This work was supported by a grant from the Norwegian Cancer Society The suspension of heat killed *S typhosa* was kindly prepared by Mrs. Gunvor Fretheim the Gade Institute Department of Microbiology

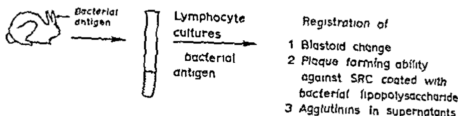


Fig 1

Schematic drawing with an outline of the experimental programme

Immunization

Four adult albino rabbits were immunized against *S typhosa* First 0.5 ml of saline suspension containing 10 bacteria was given intravenously followed by 1 ml (2×10^7 bacteria) twice weekly for four weeks Thereafter one injection weekly was given for 6 weeks followed by four bi weekly doses Three booster-doses were then given in the course of one week and the rabbits were bled 5 or 6 days after the last dose At the time of bleeding the serum agglutinin titres against *S typhosa* lipopolysaccharide coated SRC varied from 1000 to 8000 Lytic antibodies were found in titres from 500 to 4000 No agglutinins and lytic antibodies (anti SRC) in low titres only were found in the sera from the control rabbits

Culture Technique

Cell suspensions for culturing were prepared as previously described (Lamvik 1968 a) from the four immunized rabbits and from two control ones Each culture tube contained 2.5×10^6 cells in 2.5 ml of medium composed of Parker's tissue culture medium (TC199) with 20 per cent pooled normal rabbit serum The culture tubes were stimulated at the start with heat killed *S typhosa* in doses ranging from 10^9 to 10 bacteria suspended in 0.5 ml of TC199 Duplicate or quadruplicate tubes were stimulated with each dose Duplicate culture tubes were supplied with 0.5 ml of TC199 without stimulator Some culture tubes were harvested after 5 days incubation Others were cultured for 8 days with a change of medium on the fourth day

Tests for Immune Reaction

The cells used for culturing from immunized and non immunized rabbits were tested before incubation for plaque forming ability against SRC coated with *S typhosa* lipopolysaccharide as described below At the time of harvesting the culture supernatants were separated from the cells and stored at −20 C and later checked for agglutinins against SRC coated with lipopolysaccharide The harvested cells were washed once in 5 ml of Ringer solution and suspended in 0.2 ml of TC199 The suspended cells were then mixed in equal volumes with a 20 per cent suspension of SRC coated with lipopolysaccharide with 20 per cent fresh guinea pig serum in TC199 The mixtures were tested in micro incubation chambers for the formation of lytic plaques in the mono layers of SRC formed on the bottom of the chambers (Lamvik 1968 b)

The degree of cell transformation in the culture was estimated in acridine orange stained cell suspensions transferred to incubation chambers The cells were classed as small lymphocytes intermediate cells and blastoid cells according to morphological criteria defined previously (Lamvik 1968 b) The second group probably comprised partly transformed lymphocytes as well as some of the progeny of blastoid cells including some cells with a morphological appearance like that of plasma cells Permanent cell preparations were made in a sedimentation apparatus (Bøyum, Hjort & Schelberg 1964) fixed and stained with May Grunwald Giemsa

The culture supernatants from the time of harvesting and from the change of medium were tested for agglutinins by titration against lipopolysaccharide coated SRC (1 per cent in saline) either directly or following concentration to 1/3 volume using polyethylene glycol The titrations were performed in Perspex trays and read from the settling pattern

RESULTS

No plaque forming cells and no agglutinins against lipopolysaccharide coated SRC were found in the cell suspensions prepared from immunized and non immunized rabbits Less than 5 per cent partly transformed cells and no blastoid cells were found in these cell suspensions After four days culturing when the medium was changed in tubes which were harvested after 8 days there were still no agglutinins present

When the culture tubes were stimulated with 10^6 or 10^8 killed bacteria and harvested after 5 days many transformed cells including blastoid cells were found in the cultures containing cells from the immunized rabbits A bacterial dose of 10^4 or less gave no definite morphological response Despite signs of blastoid transformation in the cultures harvested after 5 days incubation only very few plaque forming cells were found No agglutinins were present in the culture supernatants

In the culture tubes harvested after 8 days incubation a clear difference was observed between non stimulated and antigen stimulated cultures of cells from immunized rabbits The response towards stimulation was dose dependent From 25 to 40 per cent transformed cells including from 3 to 12 per cent blastoid cells were found in the culture tubes stimulated with 10^8 or 10^6 killed bacteria (Table 1) When the stimulating dose was 10^4 or less the response was slight with 10 to 15 per cent enlarged lymphocytes without definite blastoid change The non stimulated cultures contained no blastoid cells but some partly transformed cells were present

TABLE 1

Blastoid Cells in Antigen Stimulated Cultures Containing Blood Lymphocytes from S typhosa Immunized and Non Immunized Rabbits Harvested after 8 Days Culturing

Stimulating dose		Blastoid cells %				
		10^8	10^6	10^4	10	None
Immunized rabbits	1		3 0	0 25	0 5	0
	2		3 75	0	0	0
	3	6 0	3 5	0		0
	4	11 5	5 0	0		0
Non immunized rabbits	1		0 75	0	0	0
	2	0	0	0	0	0

Means of result from duplicate culture tubes

Plaque forming cells (Fig 2) were found in all culture tubes containing cells from immunized rabbits harvested after 8 days when a stimulating dose of 10^8 bacteria had been added at the start of culturing (Table 2) No plaque forming cells were present in non stimulated cultures or in the cultures of cells from non immunized rabbits

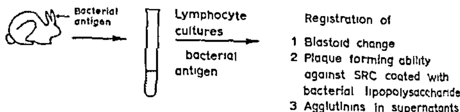


Fig 1

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Tests for Immune Reaction

The cells used for culturing from immunized and non immunized rabbits were tested before incubation for plaque forming ability *against* SRC coated with S typhosa lipopolysaccharide as described below At the time of harvesting the culture supernatants were separated from the cells and *stored at* −20 C *and later* checked for agglutinins against SRC coated with lipopolysaccharide The harvested cells were washed once in 5 ml of Ringer solution and suspended in 0 2 ml of TC199 The suspended cells were then mixed in equal *volumes* with a 20 *per cent* suspension of SRC coated with lipopolysaccharide with 20 per cent fresh guinea pig serum in TC199 The mixtures were tested *in micro incubation* chambers for the formation of lytic plaques in the mono layers of SRC formed on the *bottom of the* chambers (*Tamoil* 1968 b)

The degree of cell transformation in the cultures was estimated *in acridine* orange stained cell suspensions transferred to *incubation* chambers The cells were classed as small lymphocytes intermediate cells and blastoid cells according to morphological criteria defined previously (*Tamoik 1968 b*) *The second* group probably comprised partly transformed *lymphocytes* as well as some of the progeny of blastoid cells including some cells with a morphological appearance *like that* of plasma cells Permanent cell preparations were *made in a sedimentation* apparatus (*Bots Wint & Schaberg* 1964) fixed and stained with May Grunwald Giemsa

The culture supernatants from *the time* of harvesting and *from the* change of medium were tested for agglutinins by titration against lipopolysaccharide coated SRC (1 per cent in saline) either directly or following concentration to 1/3 volume using polyethylene glycol The *titrations* were performed in Perspex trays and read from the settling pattern

incubation chambers where they aggregated around possible antibody liberating cells and seemed to prevent these antibodies from diffusing out into the red cell monolayers thus preventing plaque formation

The appearance of agglutinins in the culture supernatants correlates well with the development of plaqueforming cells as agglutinins (titres up to 4) were only found in the cultures of lymphocytes from immunized rabbits stimulated with an antigen dose of 10^6 bacteria Supernatants concentrated 1/3 showed a higher agglutinating ability corresponding to the increase in protein concentration (Table 3) No agglutinins were detected in concentrated supernatants from control culture series or in the supernatants from the reacting culture tubes after 4 days incubation

TABLE 3

Agglutinins Against SRC Coated with S typhosa *Lipopolysaccharide in Concentrated Culture Supernatants from Antigen Stimulated Cultures Containing Blood Lymphocytes from Immunized and Non Immunized Rabbits Stimulating Dose 10^6 Killed Bacteria*

Culture time		Agglutinin titres 4 days	8 days
	1	<1	2
Immunized	2	<1	4
rabbits	3	<1	[illegible]
	4	<1	8
Non immunized	1	<1	<1
rabbits	2	<1	<1

Supernatants from duplicate culture tubes were pooled and concentrated to 1/3 volume using polyethylene glycol

Cultures showing plaque forming cells or agglutinins against lipopolysaccharide coated SRC were checked for plaque forming ability and agglutinins against SRC with negative results

DISCUSSION

The present experiments demonstrate the development of specific reacting plaque forming cells and agglutinins in blood lymphocyte cultures following in vitro stimulation with bacterial antigen The rabbits that supplied the lymphocytes were bled 5 or 6 days after the last of three booster injections following a long course of immunization No plaque forming cells were found however in the cell suspensions used for culturing

The culture experiments showed that cell transformation with blastoid cells and some cells like plasma cells occurred when the cultures were stimulated with the priming antigen No such transformation was seen when the antigen was added to cultures of non primed

lymphocytes. Cells with specific lytic activity against antigen coated SRC were found in the antigen stimulated cultures of primed lymphocyte after 8 days culturing, and agglutinins against similarly treated SRC were present in the culture supernatants. The development of plaque forming cells as well as agglutinins is thus dependent on *in vivo* priming and *in vitro* antigen stimulation.

The numbers as well as the size of the lytic plaques found in the test chambers were smaller than the numbers and size of the plaques previously registered in most culture experiments using lymphocytes from SRC immunized rabbits (*Jamvil* 1968c). The small size of the plaques is comparable to the findings reported by *Cunningham, Smith & Mercer* (1966) who used the same technique for testing cells from sheep lymph nodes and efferent lymph for plaqueforming ability against SRC coated with S. *munchen lipopolysaccharide*. The agglutinins found in our cultures were also present in very low titres in some culture tubes detectable only in the undiluted supernatant. The main reason for these low titres is probably that the *in vitro* conditions for cell growth and differentiation are inferior to those in the lymphoid organs. The low titres may in addition partly be explained by low cell numbers and relatively large volumes in the culture tubes.

The immune reaction found in our cultures is clearly different from the spontaneous *in vitro* liberation of antibodies from peripheral blood leucocytes reported by *Landy et al.* (1964) and by *Hulliger & Sorkin* (1963, 1965). This antibody liberation from leucocytes without antigen stimulation *in vitro* is probably due to the escape of antibody producing cells from the lymphoid organs into the peripheral blood. The results also appear to differ from those obtained by *Wesslen* (1952) and by *Hollander & Danielsson* (1962) on the liberation of antibodies against S. *typhosa* and horse serum from thoracic duct lymphocytes taken from rabbits after immunization and incubated *in vitro* without *in vitro* stimulation. No evolution of plaque forming cells or liberation of agglutinins was noticed in our cultures without *in vitro* stimulation.

In the present experiments the signs of specific antibody liberation developed between the 4th and the 8th day of culturing. Correlated with the lack of immune reaction in non stimulated cultures, this evidence is indicative of *in vitro* formation of antibody producing and liberating cells in the cultures and the evolution of such cells from resting lymphocytes that lack the ability to develop spontaneously into antibody producing cells.

The results are closely similar to the findings in SRC stimulated cultures with blood lymphocytes from SRC immunized rabbits (*Jamvik* 1968c) and appear to be similar to some of the findings reported by *Girard* (1968) on antibody synthesis by rabbit blood lymphocytes *in vitro* following stimulation by different antigens. Bacterial antigens as well as heterologous red cells are thus able to induce cell transformation with specific antibody synthesis in rabbit blood lymphocyte cul

tures The results also show that the indirect method of registration of plaque forming ability using red cells coated with bacterial lipo polysaccharides is suitable for testing cultured lymphocytes for specific immune reaction

SUMMARY

Blastoid transformation was demonstrated in cell cultures containing blood lymphocytes from typhoid immunized rabbits stimulated *in vitro* with killed *S typhosa* Plaque forming cells and liberated agglutinins against SRC coated with lipopolysaccharide from *S typhosa* were found in such cultures The blastoid response as well as the specific immune response was dependent on the stimulating dose given *in vitro* However although a strong blastoid response occurred following a very large bacterial dose this was not followed by the appearance of plaque forming cells and agglutinins possibly because the transformed cells became coated by the excess bacteria

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IMMUNODIFFUSION STUDIES ON *ESCHERICHIA COLI*

1 *Identification of O, K and H Antigens in an O6 Strain*

By

JAN HOLMGREN GÖSTA EGGERTSEN LARS A HANSON
and KNUT LINCOLN

Received 2 1 69

In 1943 *Kauffmann* reported that after elimination of heat labile envelope antigens *E coli* strains could be grouped with agglutinating antisera against the heat stable somatic antigens (20) This provided the key to the antigenic analysis of *Escherichia* The classical coli serology reviewed by *e g Kauffmann* (21) is based on three antigens designated O K and H The thermostable cell wall O antigens labelled O1 through O149 are well characterized lipopolysaccharides (26 31) Among the K antigens there is a distinction between L A and B antigens The L antigens are thermolabile *i e* destroyed by boiling while the B antigens lose their immunogenicity but retain their agglutinin fixing ability after such heat treatment The A antigens are thermo stable capsular antigens However these differences between the K antigens are not always quite distinct (37) The A and B antigens investigated so far have been found to consist of acid polysaccharides (18 29)) whereas at least some L antigens have been demonstrated to be proteins (29 34) The flagellar H antigens are of a protein nature (1 40)

Much of the work on the somatic antigens of Gram negative bacteria has been done on *Salmonella* Immunodiffusion techniques have had extensive application to *Salmonella* (*e g* 4 16 23 33 35 36 42) but such studies on *Escherichia* are not equally numerous (2 9 10 11 12 19 27, 29 34 35) Most of these investigations have been particularly concerned with the degree of heterogeneity of various bacterial extracts

The purpose of the present study was to obtain a more complete picture of the complex antigenic pattern of *E coli* than that given in the antigenic formula of *Kauffmann Knipschildt Vahlne* (21) and to

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Thanks are due to Miss *Birgitta Berglund* Mrs *Brita Drews* and Miss *Ingela Svardstrom* for skilful technical assistance
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investigate the possibilities of a differentiation between the O, K and H antigens by means of immunodiffusion methods. This characterization of *E. coli* antigens is part of a study of the bacteriology and immunology of urinary tract infections in children ([illegible], 13, 17, 43, 44).

MATERIAL AND METHODS

Bacterial Strains

The following *E. coli* type strains kindly supplied by Drs. *I. & F. Ørskov* at the WHO International Escherichia Centre, Statens Seruminstitut, Copenhagen, were used:

Seruminstitut designation	Serotype O	K	H
U5/41	1	1	7
U9/41	2	1	4
U4/41	4	3	5
Bi 7458/41	6	2a 2c	1
Su 4344/41	6	13	1
Bi 7509/41	7	1	–
G 3404/41	8	8	4
Su 4411/41	14	7	–
F 10018/41	18	76	14
F 14a	[illegible]	13	1
E 3b	75	?	5

In addition 29 *E. coli* strains isolated from children with urinary tract infections were employed. All of these were O grouped as described by *Lincoln* ([illegible]4). The O antigen groups O1, O4, O6, O7 and O18 were each represented by four strains, O75 by three strains and O3, O11, O2?, O25, O11? and O120 by one strain each.

All the aforementioned strains were of the smooth colony type. From the O18 K? H14 strain we isolated a rough colony variant designated O18 R which was also employed.

E. coli O6 K2a 2c H1 was chosen as a *model strain* for the antigenic analyses since it belongs to an O group very often found in patients with urinary tract infections (43) and is a serologically well defined strain ([illegible]1, 37) with no tendency to convert from the S to the R form.

Antigenic Preparations for Immunodiffusion Analyses

Stock suspensions were prepared in the following way: smooth colonies, the bacteria of which showed no auto-agglutination on heating, were selected as inocula. The bacteria were cultivated on 0.7 per cent nutrient agar plates incubated for 18–24 hours at 37° C. The growth was suspended in sterile saline. The stock suspensions were adjusted to correspond to 50 mg of acetone dried bacteria per ml.

Veronal buffer extract (VE antigen). Five g of acetone dried bacteria were suspended in 100 ml of veronal buffer, pH 8.6, and kept at 37° C for 24 hours. After centrifugation (3000 rpm, 30 min.) the supernate was decanted and used as antigen. This method for preparing antigen is known not to denature proteins (4). VE antigen was prepared from the model strain (O6 K2a 2c H1) and from the O? K1 H4, O? K13 H1, O14 K7 H–, and O?? K13 H1 strains.

Ultrasonic extract. The stock suspension of the model strain (O6 K2a 2c H1) was treated for 15 min. at 500 W and [illegible] kc/sec in an MSE ultrasonic disintegrator (MSE Ltd., London). The supernate obtained by centrifugation was used.

Free e press extract was prepared from the stock suspension of the model strain

(O6 K2a 2c H1) as described by *Edebo* (7) *The bacterial* debris was spun down and the supernate used as antigen

Heat extract antigen (HA) The stock suspension was heated at 100 C for 2 hours After centrifugation the supernate was used as antigen *HA preparations were made* from each of the aforementioned strains

Purified lipopolysaccharides (LPS) prepared according to *Westphal et al* (41) from *E coli* O1 O2 O4 O6 O7 O8 *O18* and O75 were also used and are referred to as O1 LPS O2 LPS etc These preparations were kindly supplied by *Drs B & K Jann* at the Max Planck Institut für Immunbiologie Freiburg In the immuno diffusion studies the preparations were employed at a concentration of about 1 mg/ml

Antigenic Preparations for Immunization

Three types of antigenic preparations were used for immunization live formalin killed *and heated* cultures of bacteria The cultivations were performed in an anti gen *free medium* (15) at 37° C for 6–8 hours attaining a density of 200–500 millions *of bacteria/ml* (viable count) The bacteria were killed by adding formalin to the *cultures* to a concentration of 0 5 per cent or by boiling the cultures in a water bath *for two hours* (heated cultures)

Antisera

Antisera were produced in rabbits weighing 2 3 kg by two series of intravenous injections of antigenic preparation The injection volumes in ml in the first series were 0 25 0 5 1 0 and 1 0 in the second 1 0 2 0 4 0 4 0 and 4 0 all given at five day intervals During the course of the immunizations the animals were bled each week and a final bleeding was taken at sacrifice ten days after the last injection

OKH antisera against *E coli* O6 K2a 2c H1 and O14 K7 H– were produced using formalin killed cultures for the first series of injections followed by injections of live cultures in the second

O antisera were produced by injecting heated cultures in both immunization series against the typed strains of *E coli* belonging to O groups 1 2 4 6 7 8 18 and 75

RKH antiserum was produced in the same way as OKH antisera except that formalin killed and live cultures of the R variant of O18 1 76 H14 (O18 R) were used

Immunodiffusion Methods

Immunological analyses were performed with the microplate double diffusion in gel technique described by *Wadsworth* (38) and with the immunoelectrophoretic and comparative immunoelectrophoretic techniques in the modifications of *Wadsworth & Hanson* (39) The electrophoretic separation was performed at a voltage of 5 V/cm during 80 min in 0 05 M veronal buffer pH 8 2 The results of the various antigenic analyses are based upon replicate experiments using *analogous* antisera from different rabbits as well as different bleedings from the same animal

As regards O grouping of *E coli* strains a simplified immunodiffusion method was employed Paper discs soaked with different O antisera were placed on a 2 mm thick agar layer in which basins were cut and filled with *heat extract* antigens of the strains to be grouped (cf 6 8 34 and experiment shown in Fig 8)

Titration of Agglutinins to E coli was performed as described by *Lincoln* (14)

Block Electrophoresis

Horizontal block electrophoresis was performed on the VF antigen *from E coli* O6 K2a 2c H1 using Sephadex G–25 (Pharmacia Uppsala Sweden) *as supporting* medium A slurry was made of the Sephadex with veronal buffer (pH 8 6 μ 0 075) and spread 3–5 mm thick on a glass plate (36 × 12 cm) Thereafter the antigen was *applied* in a 5 × 90 mm transverse basin cut in the gel The *electrophoretic* run was performed at a voltage of 6 V/cm for 16 hours whereupon *the block* was cut transversely in 3 cm strips which were eluted with the veronal buffer The fractions were tested by double diffusion and comparative *immunoelectrophoresis*. The

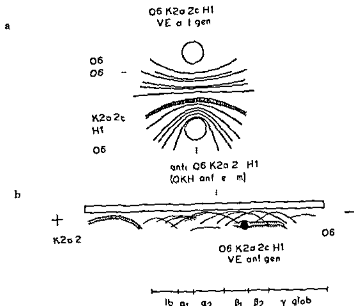


Fig 1

.matic diagram of double diffusion (a) and immunoelectrophoretl (b) analyses he model strain VE antigen developed with OKH antisera Lines formed by O nd H precipitino ens id ntified by the experiments described in this paper are . ated The electrophoretic distribution of human serum proteins is included as a reference

bohydrate content of the fractions was determined by the anthrone reaction (14) h dextran as standard Protein was determined with the *Folin Ciocalteau* reac n (25) with tyrosine as standard

RESULTS

he Antigenic Complexity of the Model Strain

By means of comparative immunological analyses of various anti enic preparations *i e* veronal buffer extract (VE antigen) ultra onic extract freeze press extract and heat extract antigen (HA) the nodel *E coli* strain (O6 K2a 2c H1) was shown to contain more than 20 separate antigenic factors The VE antigen was found to be the most representative antigenic preparation containing the largest number of demonstrable precipitinogens and was therefore chosen for the detailed analyses In this antigen at least 12 separate precipitating factors (Fig 1a) were revealed by comparative double diffusion ana lyses using different corresponding OKH antisera and other antigenic preparations from the model strain Immunoelectrophoretic studies demonstrated a good electrophoretic separation of the factors resolv ing the VE antigen into a total of at least 14–15 precipitinogens (Fig 1b) Some of the OKH antisera against the model strain lacked demon strable antibodies to one or a few of these antigenic factors

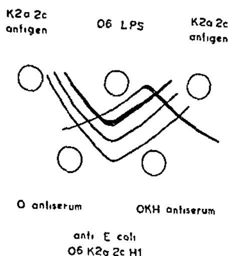


Fig 2

Schematic representation of comparative double diffusion precipitation patterns formed by the O6 LPS and the isolated K2a 2c antigens from *E coli* O6 K2a 2c H1 with an O and an OKH antiserum against *E coli* O6 K2a 2c H1

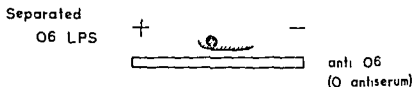


Fig 3

Immunoelectrophoretic pattern typical of those formed by the electroseparated *E coli* LPS antigens and their corresponding O antisera

Identification of O, K and H Antigens in the Model Strain

The O antigen When analysed with O6 O antisera by double diffusion three heat stable antigenic factors were demonstrable in the purified O6 LPS antigen (Fig 2) By comparative double diffusion these factors could also be demonstrated in the VE antigen from the model O6 strain using corresponding O or OKH antisera cf Fig 1a In immunoelectrophoresis the electroseparated O6 LPS developed with O6 O antisera a broad fuzzy precipitate close to the antigen basin (Fig 3) In the case of some antisera this precipitate consisted of at least two parallel lines The O6 LPS only reacted with O antisera against *E coli* O6 and not with any of the O antisera against the O groups 1 2 4 7 8 18 or 75

Experiments with the purpose of identifying the O antigen in the VE antigen preparation from the model strain were also made in which the strains of the serotypes O6 K2a 2c H1 O6 K13 H1 and O22 K13 H1 were compared with OKH antisera to O6 K2a 2c H1 Fig 4 shows two antigenic factors common to the two O6 strains

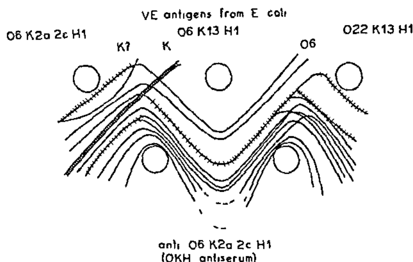


Fig 4

Comparative double diffusion analysis identifying the K and O precipitinogens in the VE antigen preparation of the model strain with an OKH antiserum against this strain The third O precipitinogen s e Fig 1a was not developed with the employed antiserum

which are not present in the O22 strain Comparative double diffusion analysis of O6 LPS and VE antigen from the model strain showed that these two factors corresponded to two of the O6 factors indicated in Fig 1a

The K antigen The comparison between the strains O6 K2a 2c H1 O6 K13 H1 and O22 K13 H1 in immunodiffusion with anti O6 K2a 2c H1 OKH immune serum illustrated in Fig 4 was used to identify the K2a 2c antigen Two lines formed with the model strain O6 K2a 2c H1 were not found to be formed with the strain O6 K13 H1 which according to the *Kauffmann Knipschildt Vahlne* antigenic formula only differs from the model strain in K antigen Another experiment with a view to identifying the K antigen was performed in which the anti O6 K2a 2c H1 immune serum was absorbed with VE antigen prepared from the strain O6 K13 H1 This absorbed antiserum should only contain antibodies to the K2a 2c antigen The VE antigen from the model strain (no 4 basin in Fig 5) was analysed with the anti O6 K2a 2c H1 OKH immune serum (no 1 basins) and with the absorbed antiserum anti K2a 2c (no 2 basins) The figure shows that only one dense precipitate possibly consisting of two lines is formed with this absorbed serum In immunoelectrophoresis the VE antigen from the model strain gave a broad and dense precipitate with the "anti K2a 2c serum This precipitate often consisting of two parallel lines was formed by the fastest moving antigenic factor (Fig 1b) The high electrophoretic mobility of this factor allowed the isolation of

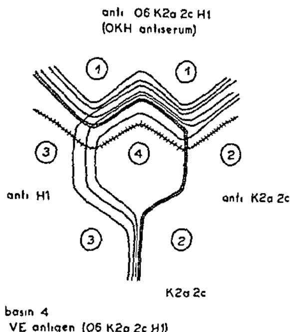


Fig 5

Immunodiffusion analysis identifying the K (right side) and thermolabile (left side) precipitinogens in VE antigen from the model strain (basin no 4) by means of anti O6 K2a 2c H1 (no 1 basins) and absorbed antisera In no 2 basins anti K2a 2c = anti O6 K2a 2c H1 absorbed with VF antigen from O6 K13 H1 In no 3 basins anti H1 = anti O6 K2a 2c H1 absorbed with HA from O6 K2a 2c H1

K2a 2c antigen uncontaminated by other antigenic material by preparative zone electrophoresis in Sephadex G-25 The K antigen containing fraction was rich in carbohydrate and could be freed from protein by *t a* precipitation with $(NH_4)_2$ SO After heating to 100° C for 2 hours the K antigen was still precipitinogenic and formed two lines with O6 K2a 2c H1 OKH antisera but no lines with O antisera By double diffusion analysis of *E coli* O6 LPS with OKH antisera against the model strain the K2a 2c antigen could be identified as a fourth factor in the O6 LPS in addition to the three factors demonstrable with O antisera (Fig 2)

The H antigen The comparison between the three strains differing in O and K antigens but having the same H antigen shown in Fig 4 illustrates that these strains have many antigenic factors in common one or more of which can be of H antigenic nature At least ten such factors were observed some eight of which lost their antigenicity after heating to 100° C for 2 hours Most of these common factors were also found in strains with other H designations (*e g* *E coli* O2 K1 H4 and O14 K7 H-)

In an effort to identify the H1 antigen the OKH antiserum to O6 K2a 2c H1 was absorbed with HA from the same strain Analysis of the VE

antigen from the model strain (no 4 basin in Fig 5) using this absorbed antiserum (no 3 basins) showed at least three antigenic factors In an attempt to making the absorbed antiserum monospecific for the H1 antigen it was further absorbed with VF antigen from the cross reacting strains O2 K1 H4 and O14 K7 H After these absorptions the antiserum formed only one line with the VF antigen from the model strain This line was presumably formed by the H1 antigen (Fig 6)

Identification of O Antigens in Other E Coli *Strains*

Immunoelectrophoretic analysis of LPS preparations from *E coli* of the O groups 1 2 4 7 8 18 and 75 with their respective O antisera showed broad precipitates of the same form and localization as the precipitate obtained with the O6 LPS and O6 O antiserum shown in Fig 3 With the exception of LPS from *E coli* O18 and O4 which cross reacted (Fig 7a and b) the LPS antigens only reacted *with their* matching O antisera

TABLE 1

O Grouping of 29 E coli *Strains Using O Antisera against Type Strains in the Simplified Immunodiffusion Technique*

E coli HA preparation		O antiserum							
O group	No of strains	O1	O2	O4	O6	O7	O8	O18	O75
O1	4	++	−	−	−	−	−	−	−
O4	4	−	−	++	−	−	−	+	−
O6	4	−	−	−	++	−	−	−	−
O7	4	−	−	−	−	++	−	−	−
O18	4	−	−	+	−	−	−	++	−
O75	3	−	−	−	−	−	−	−	++
O3	1	−	−	−		−	−	−	−
O11	1	−	−	−	−	−	−	−	−
O22	1	−	−	−	−	−	−	−	−
O25	1	−	−	−	−	+	−	−	−
O112	1	−	−	−	−	−	−	−	−
O120	1	−	−	−	−	−	−	−	−

\+ indicates faint precipitate ++ indicates dense precipitate

Further experiments with a view to establishing the O antigen specificity of precipitinogens from *E coli* were performed with the 29 O grouped *E coli* strains isolated from patients with urinary tract infections see Table 1 HA preparations from these strains of known O groups were tested with the eight O antisera anti O1 O2 O4 O6 O7 O8 O18 and O75 using the simplified immunodiffusion method (Fig 8) One dense precipitate was formed by the HA preparation from each strain and its matching O antiserum In addition the four O4 and the four O18 strains mutually cross reacted the heterologous precipitate however being fainter than the homologous These cross reactions

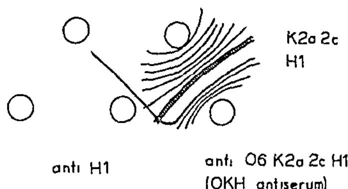
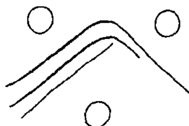


Fig 6

Comparative double diffusion analysis identifying the H1 precipitinogen in the VE antigen from the model strain using anti O6 K2a 2c H1 *and this immune serum* absorbed with the HA preparation from the model strain as well as with VE antigens *from E coli* O14 K7 H- *and E coli O2 K1 H4 The triple absorbed antiserum is* indicated anti H1



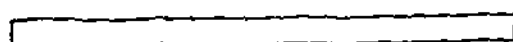


Fig 7

Comparative double diffusion (a) and immunoelectrophoretic (b) analyses showing cross reactivity *of the E coli O4 and O18 LPS antigens* using O antiserum against *E coli* O4 K3 H5

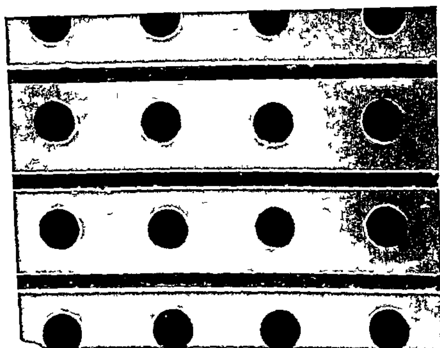


Fig 8

Photo raph of anal es with the disk immunodiffusion technique for O grouping Disks saturated with O antiserum against the O antigens from left to right O1 O? O4 O? (fir t and third rows) and O7 O8 O18 O75 (second and fourth rows) Longitud n l basins fille l with HA preparations from *E coli* strains belonging to O75 (upper ba in) O7 (middle basin) and O6 (lower basin)

were verified in microplates by comparisons with the homologous systems as shown in Fig 7a A precipitate was also formed by the O25 strain and the anti O7 O antiserum In comparative double diffusion analyses using the microplate method this precipitate was shown to give a reaction of fusion with one of the two lines formed by the O7 LPS and the anti O7 O antiserum On testing for cross agglutination the O25 strain was positive with the O7 antiserum at a dilution of 1 400 whereas the O7 strain was agglutinated at dilution 1 3 200 of this antiserum Cross agglutination between the O4 and O18 strains was also obtained

The HA preparations from the 29 *E coli* strains were also tested with the same O antisera in microplates with results corroborating those in Table 1 For practical reasons the anti O2 and O8 O antisera were excluded in these tests

Comparison of S and R Variants

Another experiment with the purpose of identifying the O antigen of *E coli* was performed in which the S and R forms of O18 K76 H14

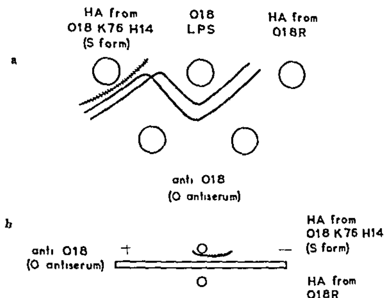


Fig 9

Comparative double diffusion (a) and immunoelectrophoretic (b) analyses showing the difference in precipitinogenic content in HA from the smooth and rough forms of *E coli* O18 K76 H14 The O antigen was not demonstrable in the preparation from the rough form

were compared Immunodiffusion analyses with O18 O antisera showed the presence of three heat stable antigenic factors in the S form of this strain Two of these factors were also present in the O18 LPS antigen but were not found in the HA preparation from the O18R strain (Fig 9a) Immunoelectrophoretic studies using the HA preparations of the S and R forms of the O18 strain with several O18 O antisera confirmed this finding a characteristic broad precipitate close to the antigen basin was developed by the S form while no line was found with the R variant (Fig 9b) Antiserum to the O18R variant (RKII antiserum) did not form any precipitate with the HA preparation of the S form

DISCUSSION

Through extensive studies using bacterial agglutination the *Escherichia* genus is serologically well defined by its O K and H antigens (21) Among the many antigenic factors revealed in the present studies of a model *E coli* strain (O6) by immunodiffusion methods some could be identified as related to O K and H antigens In addition several other antigens were common in strains which differed from the model strain in their O K and H antigens Presumably none of these common antigens corresponds to the common antigen of *Kunin Beard and Halmagyi* since the latter according to reports is not precipitinogenic (22) The M antigen (11 18 30) *might* be one of the

common antigens since it has been found to be antigenically identical in several different strains (32)

In various ways it could be demonstrated that the O6 model strain comprised precipitinogens which correspond to the O6 lipopolysaccharide In immunoelectrophoresis the O antigen appeared as a broad and dense precipitate in the region corresponding to that of the human serum β_2 globulin region The form of the precipitate indicates a poorly diffusing antigen of high molecular weight (Fig 1b) This precipitate most often consisted of two or more parallel lines with similar electrophoretic localization O antigens (LPS) purified according to *Westphal et al* (41) from *E coli* of other O groups gave similar immunoelectrophoretic patterns with their matching O antisera Consistent with the notion that these precipitates corresponded to O antigen was the observation that a similar precipitate was formed by the S form but not by the R form of an O18 strain This is in agreement with the concept of *Moller* that there is no serological relation between heat stable antigens of R and S forms of *E coli* (28) In this relation it is noteworthy that recent chemical analysis of the O antigen of the O18 strain used in the present work revealed that rhamnose was the only carbohydrate in addition to the basal carbohydrate core presumed to be the same in the R and S forms of this as well as of other coli strains (31)

The purified O6 lipopolysaccharide preparation formed three precipitates with O antisera An additional precipitate only formed with OKH antisera was due to a contamination with K antigen in the O antigen preparation (Fig 2) Whether any other of the three remaining precipitates was formed by an impurity or they were all formed by a heterogeneous O antigen cannot be settled at present Other workers (35) have also obtained more than one precipitate with purified O antigens from *E coli* These observations together with the many cross reactions observed between various *E coli* O groups in agglutination experiments (31) may be taken in support of the suggestion by *Ørskov et al* (31) that the O antigens of *E coli* are complex and composed of several factors as in the *Salmonella* O groups Such a view is consistent with the finding that two precipitinogens were common to the O4 and O18 lipopolysaccharide antigens and one was not (Fig 4) and the similar observation that one of the two precipitinogens in the O7 LPS was also found in an *E coli* O25 strain

The K antigen was identified in the VI antigen preparation from the model strain by *in* absorption experiments and because of its high electrophoretic mobility it could be obtained in a pure state by preparative zone electrophoresis Recently K antigens have also been isolated by means of fractionated precipitation with cetavlon which precipitates acidic polysaccharides (5 18 30) The two parallel lines often obtained with the K antigen in immunoelectrophoretic analyses may indicate a heterogeneity of the K antigen Such a heterogeneity

might also explain the finding of the two factors in the model strain but not in another strain which according to the Kauffmann-Knipschildt-Vahlne antigenic formula only differs in its K antigen. It cannot be definitely stated however that both of these factors really are K antigens since there may be antigens other than K and not included in the classical antigenic formula which differ in the two strains.

The K2a 2c antigen of the model O6 strain has been classified as a thermolabile L antigen (21). In spite of this we found that it retained its precipitinogenic ability after boiling for two hours. However the K2a 2c antigen native or boiled was precipitated only by OKH antisera but not by O antisera. These properties indicate that it is rather a B antigen. I. and F. Ørskov have also demonstrated K antigens of the B type in L strains of E. coli (29).

E. coli bacteria belonging to the O group 6 are often found to be the causative organism in urinary tract infections in children (44). Our earlier studies have shown that children with pyelonephritis caused by E. coli form antibodies against the O antigen of the infecting bacteria (3, 43). Preliminary experiments however have shown that such children also can form antibodies against antigens other than the O antigen (17). Thus to get a more complete picture of the immune response in patients with urinary tract infections it is necessary to obtain a more detailed knowledge of the antigenic mosaic of the infecting strains.

SUMMARY

The antigenic pattern of an E. coli O6 type strain was investigated using immunodiffusion methods. More than 20 antigenic factors could be discerned using various types of antigen preparations. O, K and H antigens could be identified. Using O6 O antisera three antigenic factors were found in the purified O6 lipopolysaccharide indicating a possible heterogeneity of the O antigen. Due to its high electrophoretic mobility the K antigen could be isolated by preparative zone electrophoresis.

By comparison of the E. coli O antigens of the O groups 1, 2, 4, 6, 7, 8, 18 and 75 using double diffusion technique a cross reaction was observed between O4 and O18; in addition O25 reacted with O7 O antiserum.

A double diffusion method using disks soaked with antiserum was applied for O grouping of E. coli strains.

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THE DISTRIBUTION OF TRITIUM-LABELLED BENZYLPENICILLIN IN STAPHYLOCOCCAL ARTHRITIS

An Autoradiographic Study in Golden Hamsters

By

LARS LINDBERG

Received 15 i 69

It is well known that it is often difficult to cure infections in the skeleton and in joints with antibiotics It appears that the substance given does not reach or does not affect the bacteria in certain parts of the focus For this reason a series of investigations about the distribution of antibiotics in infectious lesions in the skeleton and joints have been started As a link in this research programme the distribution of tritium labelled dihydrostreptomycin in experimental tuberculous osteomyelitis and of tritium labelled dihydrostreptomycin and tetracyclin in experimental staphylococcal arthritis have been described in two earlier papers (*Lindberg* 1967 *Lindberg & Lundberg* 1968) This paper in which the distribution of tritium labelled *benzylpenicillin* in experimental staphylococcal arthritis is described is a continuation on this program

MATERIAL AND METHODS

(A more detailed description of the method is found in the paper of *Lindberg & Lundberg* 1969)

Twenty golden hamsters weighing about 100 g each were injected in the left knee with 0 05 cc of a suspension of *Staphylococcus aureus* strain Wood 46 containing 10^9 colony forming units per cc This injection produces a standard arthritis described by *Lindberg* (1969) The animals were devided in two groups of ten animals each The first group was injected with tritium labelled benzylpenicillin two days and the other group two weeks after the bacterial injection

Each animal received an injection of 0 25 cc of physiological saline containing 3 09 mg of tritium labelled benzylpenicillin potassium per cc corresponding to 0 7 mCi/animal The solution was injected intramuscularly under the left scapula

Two animals of each group were killed 15 minutes 30 minutes 1 hour 3 hours and 6 hours respectively after the injection of the penicillin The infected knee joints were removed frozen in Hexane and carbon dioxide snow freeze dried em

This investigation was made possible by a grant from *Alfred Österlunds Stiftelse*

The radioactive penicillin benzylpenicillin T(G) potassium had a specific radioactivity of 0 45 mCi/mMol and was bought from The Radiochemical Center Amersham The radiochemical purity was over 93 per cent



Fig 1

Autoradiogram (top) and corresponding histological section from knee infected two weeks *previously* The animal was killed [illegible] minutes after the injection of tritium labelled benzylpenicillin The large dark areas in the autoradiogram ventrally and dorsally in the knee represent abscesses with penicillin in high concentration *The* autoradiogram was exposed [illegible] days Haematoxylin Magnification × 1[illegible]5

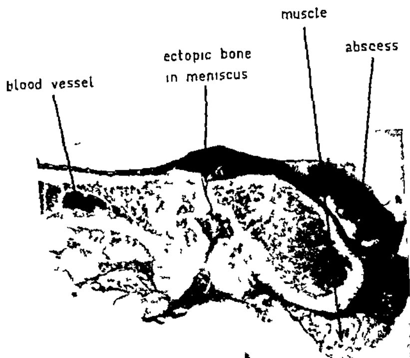


Fig 9

Autoradiogram of knee infected two days previously The animal was killed 15 minutes after injection of tritium labelled penicillin Note the high concentration in the large tibial blood vessel The autoradiogram was exposed 21? days Magnification × 12 5

bedd d and tape secti ned The tape mount d sections were fastened to Ilford G 5 autoradiographic plates and the autoradiograms exposed 7–8 months After exposure plates and sections were processed in the usual way (*Tin lberg & Lundberg* 1969) The autoradiograms and the corresponding histological sections were afterwards compared by placing the sections over the autoradiograms

RESULTS

The distribution pattern of the penicillin given two days after injection of the bacterial suspension did not differ with certainty from that found when the penicillin was not given until two weeks after the bacterial injection

The distribution of the penicillin seen in the infected knee joints of animals at different intervals after the injection are given below

Already after *15 minutes* the penicillin had diffused into all the tissues of the injected joints except the calcified bone tissue (Figs 1 and 2) The highest concentration was found in the pus of abscesses and in soft tissues penetrated by inflammatory cells where the blackening of the autoradiograms was of comparable intensity The blackening over blood vessels was somewhat lower than that over the pus and of

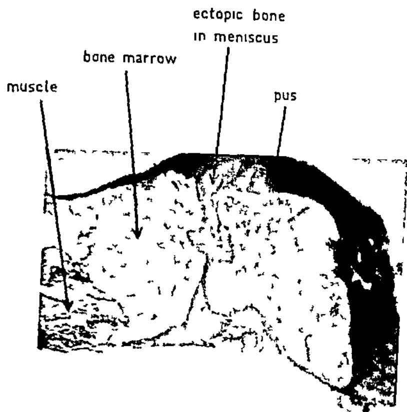


Fig 3

Autoradiogram of knee infected two weeks previously The animal was killed [illegible] minutes after injection of tritium labelled benzylpenicillin The relative concentra[illegible]tion of penicillin is the same as that 1 minutes after injection The autoradiogra[illegible] was exposed 21? days Haematoxylin Magnification × 1?5

about the same intensity as that over the periosteum and over the fibrous streaks between the muscle bundles A still lower concentration was found in joint cartilage menisci muscles and bone marrow The blackening over the epiphyseal lines was very low and resembled that over calcified bone tissue

After *30 minutes* the distribution pattern seemed to be unchanged (Fig 3)

After *1 hour* the concentration had decreased considerably in all tissues The concentration in the infected tissues was *still higher than* that in normal tissues (Fig 4)

After *3 hours and 6 hours* the concentration in all tissues was so low that no autoradiograms were obtainable

DISCUSSION

According to different authors 40–70 per cent of intramuscularly given benzylpenicillin is excreted unchanged in the urine a small part



Fig 4

Autoradiogram (top) and corresponding section from knee infected two weeks previously The animal was killed one hour after injection of tritium labelled penicillin The concentration of the penicillin is still somewhat higher in the pus in the knee joint than in other soft tissues Note that the autoradiogram has been exposed 243 days to obtain this degree of blackening For this reason it is not possible to compare the absolute blackening of this figure with that of Figs 1–3 Haematoxylin Magnification × 12 5

is also excreted with the bile whereas the remaining part 30-50 per cent is excreted in the urine as inactive metabolites (*Ullberg* 1954, *Walter & Heilmeyer* 1965). *It is difficult to obtain more exact values as these are varying with the different methods and animals used. Considerable individual variations of the values are also found when the excretion in human beings is investigated.*

In this investigation it is thus possible that parts of the blackening of the autoradiograms is caused by radioactive metabolites. As the definitive distribution pattern however is reached already 15 minutes after the injection of the penicillin this risk is very small as no large amount of the penicillin can have been broken down in such a short time (*Ullberg* 1954). The risk that the breakdown of the penicillin occurs extremely rapid in e. g. pus or inflammatory cells naturally can exist and must be borne in mind. In spite of this the method has certain advantages over *in vitro* determinations of the penicillin concentration in concentration in biopsy specimens as it is possible to assess the relative concentrations of the penicillin in the various tissues at a microscopical level without contaminating them with blood or other tissue fluids containing different amounts of the antibiotic.

Ullberg (1954) using autoradiographic technique has investigated the penetration of ^{35}S labelled benzylpenicillin into experimental abscesses produced by intramuscular and subcutaneous injections of *Corynebacterium pyogenes* in mice. The abscesses were between 6 and 30 days old. The penicillin was found to penetrate the abscess wall and was 30 minutes after the injection found in a thin layer of the abscess content immediately inside the wall. The concentration of the penicillin in this layer was higher than that in the abscess wall and was higher than or comparable to that in the blood but most part of the abscess content was totally devoid of penicillin.

In this investigation the penicillin had completely penetrated the content of all abscesses and all other infected tissues already 15 minutes after the injection. Moreover a higher concentration was found in these tissues than in the blood and in other tissues as long as autoradiograms could be obtained.

As to the penetration of benzylpenicillin into the abscesses the difference in the results obtained by *Ullberg* and the results of this investigation can be caused by several factors as e. g. differences in the age, the content or the wall of the abscesses. However the results do not allow any conclusions about this question.

The results from both investigations however show that benzylpenicillin has a tendency to be concentrated in pus. The reason for this is not known. As the concentration in the pus was higher than that in the blood or any other part of the histological section it seems reasonable to assume that the penicillin (or metabolites of the penicillin) in some way was bound to the constituents of the pus. Diffusion alone cannot explain this increase of concentration from blood to pus. The

same tendency was found for dihydrostreptomycin both in pus from staphylococcal arthritis and in tuberculous pus (*Lindberg* 1967 *Lindberg & Lundberg* 1969) where the high concentration was shown to depend on a reversible binding of the dihydrostreptomycin to cellular remnants stainable with haematoxylin probably nuclear remnants

SUMMARY

The distribution of benzylpenicillin in experimental staphylococcal arthritis has been studied with autoradiographic technique After intramuscular injection the penicillin is found to penetrate into the infected tissues even abscesses It is also found to be concentrated in pus compared to normal soft tissues and blood The reason for this concentration is discussed

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BRIEF REPORT

PEROPERATIVE EVALUATION OF THE JUXTAGLOMERULAR APPARATUS IN HUMAN KIDNEYS

A Rapid Frozen Section Technique Especially Applicable to Hypertensive Patients

By *Poul Faarup* and *Michael Petri*

In human hypertension structural changes in the juxtaglomerular apparatus (JGA) of the stenosed kidney have been found to be of diagnostic interest (e g *Genest et al* 1966 *Faarup et al* 1967 1969)

In a number of cases it may be undesirable to run the risk of the pre operative needle biopsy Thus a peroperative histological method by which the occurrence of hyperplasia or hypergranulation of the epithelioid cells in the JGA could be rapidly estimated in a peroperative biopsy has been considered clinically important. In cases with segmental lesions biopsies may aid the surgeons in localizing the involved segment during the surgical procedure

Crocker (1964) has shown that the use of general tissue stains on frozen sections may demonstrate cases *of pronounced hyperplasia of the JGA In such preparations* however the identification of the juxtaglomerular cell types is difficult and the *juxtaglomerular granules will remain unstained Therefore* a staining method which gives a reliable definition of these structures has been worked out

Technique

Cryo technique Unfixed kidney tissue was frozen in a mixture of isopentane and dry ice (app —65 C) *or in isopentane cooled by liquid nitrogen* (app —160 C) Thin sections were cut on a freezing microtome or—preferably—on a cryostat

Staining procedure A number of quantitative variations of the periodic acid Schiff method was tried in order to speed up the procedure It was found that a *reliable and reproducible method for frozen sections was obtained by halving the* time traditionally used while keeping the fluids in constant agitation by a magnetic stirrer

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Figs 1–4

Fig 1 In the wall of the afferent arteriole (AA) several epithelioid cells with *juxtaglomerular granules* may be seen (arrows) MD Macula densa EA Efferent arteriole (C Cell group of Goormaghtigh (Frozen section PAS × 490)

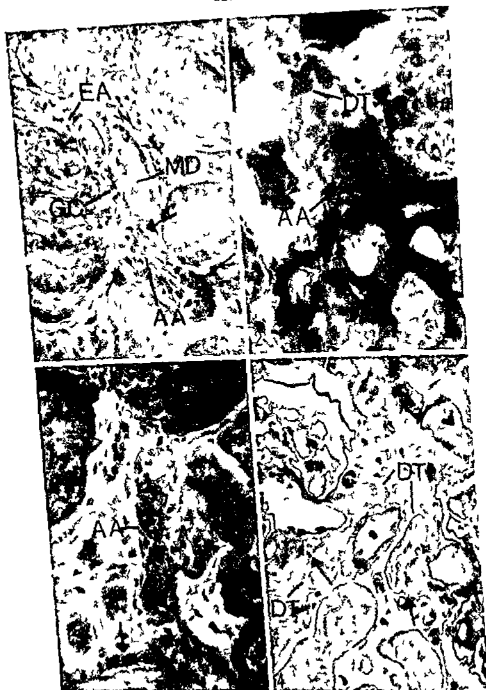
Fig 2 Tangential section of an afferent arteriole (AA) in which juxtaglomerular *granules are easily identified in the cell* DT Distal tubule (*Frozen section* PAS × 710)

Fig 3 Juxtaglomerular *granules are found in most epithelioid cells of the afferent* arteriole (AA) Epithelioid cells occur in the *pre afferent arteriole as well* (arrow) Moderate *interstitial fibrosis is present (Frozen section* PAS × 560)

Fig 4 Granulated epithelioid cell (*arrows*) *in two* juxtaglomerular apparatus close to the distal tubule (DT) The glomerular and tubular basement membranes are well defined (Paraffin section PAS × 480)

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EA
MD
GC
AA
DT
AA
AA
DT
DT

Schiffs reagent was prepared according to the "cold" Schiff *procedure of Lillie* (1965). Counterstaining was done by *celestine blue picric acid after Petri* (1968). The contrasting green colour obtained greatly facilitates the recognition of PAS positive structures such as juxtaglomerular granules.

Sulphite rinses (0.5 per cent) were prepared fresh from a 10 *per cent* stock solution of sodium metabisulphite.

		Time
1	Periodic acid 1 per cent	5 min
2	Rinse in tap water	[illegible] min
3	Schiffs reagent	[illegible] min
4	Sulphite rinse 0.5 per cent	3 × [illegible] min
5	Rinse in tap water	[illegible] min
6	Celestine blue	[illegible] min
7	Saturated aqueous picric acid	[illegible] min
8	Dehydrate quickly by absolute ethanol clear mount in DPX	1 min
	Time of staining procedure	25 min

Results

While the 25 minute frozen section procedure is being carried out one may of course do a general tissue stain of short duration on a few sections. We found van Gieson's stain well suited for estimating general kidney morphology. However the PAS method gives a differentiation of the general kidney structure which is superior to general tissue stains used for routine diagnostic work. Thus the different tubular segments of the nephron as well as glomerular and arterial morphology are well demonstrated. In Fig. 1 a JGA from the stenosed kidney of a patient with renovascular hypertension is seen. In the distal part of the afferent arteriole and in the Goormaghtigh cell region a large number of epithelioid cells can be identified. In some of these cells the juxtaglomerular granules are recognized. In thin sections it is possible to demonstrate single granules in the cytoplasm (Fig. 2).

The results of the present method applied to surgical cases of hypertension will be published separately (*Faarup et al.* 1969).

In connection with the present studies on a method for rapid evaluation of the juxtaglomerular apparatus in peroperative biopsies we investigated the value of the modification of the PAS method previously described by *Petri* (1968) for *Helly fixed paraffin embedded tissue*. A definition of histological details in the JGA was better (Fig. 4) than that obtained with the Bowie stain. The latter is a somewhat more tricky and less reproducible method and even more time consuming. Besides with the PAS method the different cell groups are well distinguished, cellborders are more easily seen and the juxtaglomerular granules stand out in a bright red colour. It should be mentioned however that in some species of animals, e.g. in rats the PAS method for the JGA is inferior to Bowie's method.

Summary and Conclusions

By the frozen section PAS method described a rapid clinically useful staining method has been devised for peroperative kidney biopsies particularly well suited for estimating alterations in the juxtaglomerular apparatus. The method was found suitable also for paraffin embedded tissue preferably fixed by *Helly's fluid*. In addition in both frozen and fixed tissue general kidney morphology is well differentiated including important diagnostic details, *e.g.* the glomerular basement membrane.

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TRANSACTIONS OF THE MEDICAL MICROBIOLOGY DIVISION OF THE SWEDISH MEDICAL SOCIETY

Annual Meeting 28th November–1st December 1968

IMMUNOLOGY

Vera Beckman & *G Möller* Transplantationlaboratory The Seraphimerhospital Stockholm SUPPRESSION OF LYMPHOCYTE-MEDIATED CYTOTOXICITY *IN VITRO* BY ANTISERA AGAINST HUMAN GAMMAGLOBULINS

In our experiments rabbit antisera against different human γ globulin classes (IgG IgA and IgM) were used With each of these antisera it was possible to demonstrate a weak stimulation of the DNA synthesis in vitro in human lymphocytes from peripheral blood in 2 experiments out of 7 performed The maximum of the stimulation was reached on the fifth day In the other experiments these antisera had no demonstrable effect on the DNA synthesis Furthermore studies of the cytotoxic effect of human lymphocytes against human fibroblasts *in vitro* were performed The lymphocyte were added to monolayers of fibroblasts on pre indicated sites where the fibroblasts get damaged and give a clear plaque which is graded from + to ++++ Normal lymphocytes were induced by phytohaemagglutinin (PHA) to kill allogenic fibroblasts and this cytotoxic effect could be partly or totally suppressed by each of these antisera In the same dosages these antisera alone without the presence of PHA could not be shown to induce any cytotoxicity in the lymphocytes against fibroblasts

B Bloth & *S E Svehag* Department of Immunology National Bacteriological Laboratory Stockholm ULTRASTRUCTURE OF PAPAIN DIGESTED IgM FRAGMENTS

To be published in J Exp Medicine 1969

O Strannegård The Department of Virology Institute of Microbiology University of Gothenburg REAGIN RESPONSE IN RABBITS FOLLOWING PARENTERAL AND ORAL ANTIGEN ADMINISTRATION

Formation of reaginic (homocytotropic) antibodies was obtained in rabbits following immunization with bovine serum albumin (BSA) or dinitrophenylated BSA (DNP BSA) By means of chromatographic experiments and absorptions with specific anti heavy chain and anti light chain sera strong evidence was obtained that the rabbit reagins belong in an immunoglobulin class which is distinct from IgG IgA and IgM

The initial logarithmic phase of the primary reagin response proceeded rapidly indicating a doubling time of 7 8 hours The anti DNP reagins generally appeared earlier and disappeared more rapidly from the circulation than did the anti BSA reagins After booster antigen injections a secondary type of reagin response was evoked in some cases This response was more readily obtained in rabbits with low

serum titres of agglutinating antibodies than in those with high titres suggesting the possible existence of antibody induced suppression of reagin response

Following oral administration of antigen a reagin response was evoked in one out of 13 rabbits which had been fed BSA and in one out of 13 rabbits fed egg albumin The serum titres of anti BSA reagins persisted for several months following cessation of antigen administration

Hederstedt B Lind J & Lundblad C National Bacteriological Laboratory Stockholm STUDIES ON HEAT AGGREGATION OF SERUM PROTEINS

Normal human sera were heated up to 40 C–63 C for varying time intervals Sephadex G–200 gel filtration of heated sera revealed a protein rise of the 19S peak and a decrease of the 7S and albumin peaks In the case of a serum heated at 63 for 10 minutes at 56 for 30 minutes or at 40 C for 5 days the 19S peak increased by 140 80 and 20 per cent respectively

With gel diffusion precipitation lines were formed between material from the increased 19S peaks and anti IgG and anti albumin sera Using a 390 cm column of Bio Gel A–15 m the macromolecular material was separated further and heat aggregation of IgM and IgA was demonstrated

Diluted syphilis sera of high TPI and FTA antibody titres (IgG only) were heated at 56 C for 24 hours Sixty five per cent of the 7S globulins were aggregated which led to a decrease of more than 2 two fold TPI titre steps but only one FTA titre step Neither TPI and FTA antibodies nor anti complementary activity was demonstrated in the enlarged 19S peaks The following questions were raised May antibodies be heat inactivated without being aggregated? Are certain IgG antibodies more readily aggregated than others?

VIROLOGY AND CELL BIOLOGY

Marianne Forsgren Central Bacteriological Laboratory of Stockholm City
ECHOVIRUS 6 ANTIGENS

The antigen composition of echovirus 6 preparations has been studied with immunodiffusion immunoelectrophoresis staining of precipitates with the nucleic acid staining acridine orange antigen separation in CsCl density gradient complement fixation and electronmicroscopy Precipitation against human convalescent sera and acridine orange staining of the precipitates show that native preparations of echovirus 6 contain two different antigens one heat labile RNA staining antigen (N) and one heat stable RNA negative (H) antigen In immuno electrophoresis at pH 8 2 (agarose) the two antigens migrate at equal velocity at pH 7 0 the N antigen migrates slower than the H antigen In CsCl density gradient a separation of the two antigens is achieved one band (density 1 [illegible] g ml) carries max infectivity complement fixing and precipitating activity of N character The other band (density 1 30) carries low infectivity complement fixing and precipitating activity of H character Electron microscopy (carried out by Bjorn Bloth Immunol Dep SPL) shows complete virions in the N band and empty capsids in the H band

Antibodies against both the N and H antigens are developed during echovirus 6 infections in man

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This study was supported by grants from the WHO

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J Anherst & H O Sjögren Department of Medical Microbiology University of Lund Lund Sweden CROSS REACTION BETWEEN TUMOUR ANTIGENS OF ADENO 7 AND 12 SARCOMAS

With a Cr 51 cytotoxic test developed to work on nonlymphoid target cells hamster adeno 7 and 12 tumours were shown to be sensitive to the tumour specific cytotoxic antibodies of mouse antisera against syngeneic adeno 12 tumours When the same sera were tested against control hamster cells (BHK-C13) no antibody activity could be detected the isotope release being similar with active and with inactive complement The cytotoxic effect on hamster adeno 7 tumour cells was confirmed by the colony inhibition technique Furthermore it was demonstrated by transplantation tests in mice that the hamster adeno 7 tumour cells were immunogenic causing an isograft immunity to adeno 12 tumours similar to that induced by adeno 12 hamster and mouse tumour cells

G Wadell Karolinska Institutet Stockholm HAEMAGGLUTINATION WITH ADENOVIRUSES BELONGING TO ROSEN S SUBGROUPS II AND III

These adenoviruses have previously been shown to agglutinate rat red cells completely and partially respectively The haemagglutination with *purified* virions and crude virus preparations enhanced by heterologous antisera of serotypes 1 2 4 5 6 (III) and 9 15 (II) was studied With all these preparations complete agglutination of *rat* red cells and also haemagglutination of *human* O was demonstrated

Experiments concerning adsorptions of virions by red cells competitive *interaction* of incomplete haemagglutinins (HA s) (i e pentons and fibres) with the agglutination by virion associated HA and the effect of *receptor destroying* enzyme on red cells were performed The results suggest that *haemagglutination* patterns were dependent of the relation between *incomplete and complete* HA s the number of receptors on the red cells and possibly also qualitative differences between the receptors

Haemagglutination of rat cells at 20 C by the tested *serotypes* was considered more efficient than the use of human O red cells *since agglutination* of the latter was more sensitive to the presence of *incomplete HA s* The former system was used to determine the optimal antigen *preparations* of serotypes belonging to subgroup III for haemagglutination inhibition *tests* *Unfractionated* virus preparations containing heterologous antisera were recommended

Pettersson Ulf & Höglund S Department of Microbiology The Wallenberg Laboratory and The Institute of Biochemistry *Uppsala* PURIFICATION AND CHARACTERIZATION OF PENTON ANTIGEN FROM ADENOVIRUS TYPE 2

The penton antigen from adenovirus type 2 has been purified by freon *extraction* high speed centrifugation *exclusion* chromatography *on* 6 per cent agarose and preparative polyacrylamide electrophoresis

The pure product sediments as a *homogeneous* peak with an S value of 10 7 ± 0 3 and analytical polyacrylamide electro*phoresis* and immuno electrophoresis reveal one single component

Immunodiffusion shows that the vertex capsomer is mainly subgroup specific while the fibre contains two specificities *one subgroup* and one type specific

The pure penton acts as an *indirect* haemagglutinin in the presence of *hetero*typic penton antibodies but is in *the order* of 5 times less *effective* than the fibre on a weight basis as could be expected from the difference in molecular weight

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Pure penton induces cytopathic effect at a level of 0 1 μg penton/10^6 KB cells This effect could be blocked by antisera against pentons but not by antisera against fibres

Trypsin abolishes the cytopathic effect of the penton completely but leaves part of the antigenicity of the vertex capsomer intact

Pure penton induces high titres of neutralizing antibodies when measured by the fluorescent focus assay but not when measured by the plaque assay

Amino acid analysis shows that the primary structure of the vertex capsomer must be different from that of the hexon provided both structures are composed of one single polypeptide chain

Electronmicroscopy reveals the classical structure composed of a spherical part with a central hole—the vertex capsomer—and the elongated fibre

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BACTERIOLOGY

H O Hallander Kathrine Dornbusch & G Laurell Department of Medical Microbiology University of Uppsala Uppsala Sweden DETERMINATION OF METHICILLIN RESISTANCE INFLUENCE OF STAPHYLOCOCCAL HETEROGENEITY ON THE DISC DIFFUSION METHOD

In this study 49 methicillin resistant penicillin β lactamas producing strains of *Staphylococcus aureus* (met p) selected according to *Barber & Waterworth* (1) were compared to 50 methicillin sensitive but still penicillin β lactamas producing strains (met p) All met p strains were shown to be heterogeneous with a very constant frequency of about 1 cell per 10^6 resistant to 400 μ and 1 cell per 10^4 resistant to 12 5 μ methicillin Colonies selected from plates with 400 μ methicillin quickly resegregated when cloned on ordinary medium

In a strictly standardized disc (30 μ) diffusion test with ordinary sheep blood agar medium and incubation at 37 C according to *Ericsson et al* (2) 40 per cent of the resistant strains were falsely negative The zone of inhibition varied from 0 to 25 mm None of the met p variants gave a zone less than 26 mm

Because of the very constant heterogeneity this variation must be due to factors in the disc diffusion method unfavourably affecting the relatively instable methicillin resistant mutants

A somewhat better separation between the two groups of strains was demonstrated with weaker discs (10 μ)

When the medium was osmotically stabilized with 5 per cent NaCl the zones for met p bacteria were on an average 4 5 mm less with no falsely negative results No change at all could be shown for met p strains

The best results were obtained when the strains were incubated on ordinary sheep blood agar medium at 30 Now the inhibition zone were on an average 19 5 mm less for met p with no change for met p variants

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Carl Ericson Institute of Clinical Bacteriology Allmänna sjukhuset, Malmo

RESISTANCE TO ACRIFLAVINE AND CADMIUM AND CHANGED PHAGE REACTIONS – MARKERS OF A NEW STAPHYLOCOCCAL PENICILLINASE PLASMID?

Staphylococcus aureus strain Fr 837–2 produced penicillinase (*pen*) and was resistant to several antibiotics It was resistant to acriflavine (MIC 200 μg/ml) and Cd (MIC 8 0 × 10 5 M) but sensitive to Hg (MIC 2 3 × 10-5 M) It was non typable (NT) with standard phages at 1000 × RTD

Penicillinase negative (*pen*) variants were isolated on Water blue agar (0 01 per cent water blue Merck Darmstadt) containing 0 05 μg/ml methicillin After 2 days at 37 C and flooding with 3 per cent benzyl penicillin *pen* colonies turned dark blue

Pen clones were sensitive to acriflavine (MIC 12 5 μg/ml) Cd (MIC 2 4 × 10 6 M) and Hg^2 (MIC 2 3 × 10-5 M) and had the phage type 7/47/54/75 at RTD but otherwise seemed identical with the parent strain No difference in lysogenicity could be shown

All markers i e penicillinase production the NT state resistance to acriflavine and cadmium were segregated together and at increased frequencies on treatment with acridine orange (25 μg/ml) or incubation at 42 C

The change of phage reactions was from inhibition reactions at 1000–10 000 × RTD to plaques at RTD There may be some analogy between this effect and loss of plasmid induced phage restriction in *Enterobacteriaceae*

As yet the data point to acriflavine resistance and changed phage reactions as probable markers previously not described of a penicillinase plasmid

Transduction experiments are under way

Paula Branefors & Pål Henry Jeppson Department of Bacteriology and the Department of Ear Nose and Throat Diseases Sahlgrenska Sjukhuset Goteborg

HAEMOPHILUS INFLUENZAE AND ACUTE EPIGLOTTITIS – BACTERIOLOGICAL AND SEROLOGICAL STUDY

Six patients four children and two adults with the clinical diagnosis of acute epiglottitis were studied from bacteriological and serological points of view

Haemophilus influenzae type b was isolated from the epiglottic region in all the patients Blood cultivation before antibiotic treatment showed *H influenzae* type b in five cases

Blood samples for the serological analyses were taken on admission to hospital after one week and after three weeks The serological analyses were performed with the double diffusion in gel technique Free capsular substance b was demonstrated in the first serum sample of three patients and was considered as a sign of massive bacteraemia Capsular b antibodies could not be demonstrated in the first serum sample from any patient and only irregularly in the second and third samples All patients however had precipitating antibodies against the cell wall antigen (O antigen) even in the first serum sample

It is therefore possible that a local allergic reaction superimposed on the inflammatory reaction caused by the bacterial invasion results in the often excessive swelling of the epiglottic tissue

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Inna Stina Malmborg S O Liljedahl B Nyström Sig Seim & I Sjöberg Surgical Clinic and Laboratory for Clinical Bacteriology Karolinska Hospital and the National Bacteriological Laboratory Stockholm INFECTIONS WITH PSEUDOMONAS IN A BURNS UNIT

The contamination of burns and of the ward environment with *Pseudomonas aeruginosa* was studied in a burns unit for 6 months

The study comprised 41 patients *Pseudomonas aeruginosa* strains were isolated from 17 of the patients mainly from patients with extensive burns Only a few of these patients had clinically observable infections This is probably mainly due to the principles of treatment open treatment with warm dry air and restrictive antibiotic therapy *Pseudomonas aeruginosa* strains were also isolated from the ward environment mainly from wash basins and sinks

Fifty three phage patterns were found among 255 pseudomonas strains Five phage patterns of similar type were grouped together These 5 patterns comprise 47 per cent of the isolated strains

An attempt was made to control the contamination of the environment by routine use of Sanivit a detergent for cleaning wash basins and sinks The detergent is bactericidal due to release of chlorine The introduction of this hygienic procedure was followed by a temporary reduction in the number of strains isolated from the environment The number of strains isolated from the patients was considerably reduced

H Cnorpe & I Olding The Institute of Medical Microbiology Department of Bacteriology and The Institute of Pathology University of Uppsala Uppsala RETROGRADE PROTEUS PYELONEPHRITIS IN RABBITS

In Proteus induced urinary tract infections the urinary findings of leucocytes are often sparse and irregular Earlier in vitro experiments have shown that leucocytes are rapidly disintegrated in alkaline media i e when Proteus organisms grow in urine

This work was done to test these findings in experimental pyelonephritis Rabbits were given retrograde pyelonephritis with a method modified from Frat The urinary findings of leucocytes bacteria and the urinary pH were studied After sacrifice urine and kidney cultures were made and the microscopic picture of the inflammatory changes in the kidneys were studied

In 75 per cent of the rabbits infected with Proteus there was a tendency to urinary pH levels above 8 0 The urinary findings of leucocytes in those cases were sparse in spite of pronounced inflammatory reaction in the kidneys In 25 per cent of the Proteus infected cases there was no rise in the urinary pH and large numbers of leucocytes were found in the urine

With the aid of fluorescent antibody technique the bacteria were found to be localized interstitially in the kidneys

J Holmgren G Eggertsen Lars Ake Hanson & K Lincoln Institute of Medical Microbiology University of Göteborg Göteborg Sweden STUDIES ON THE K AND H ANTIGENS IN AN E COLI O6 TYPE STRAIN

An *E coli* strain serotype O6 K?a ?c H1 was studied by immunodiffusion methods with the aim of identifying the K?a c and the H1 antigens From previous investigations this strain used as med I strain, was known to contain more than 8 antigenic factors three of which were related to the O6 antigen

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By comparison in double diffusion of the model strain and an O6 K13 H1 strain and by absorption of anti O6 K2a 2c H1 immune serum with the last mentioned strain the K2a 2c antigen *of the model strain was identified* In *immunoelectrophoretic analysis the* K2a 2c antigen was found to be the fastest migrating antigenic factor in the model strain This made it possible to isolate it by means of preparative zone electrophoresis in Sephadex G-25 The isolated K antigen could sensitize untreated sheep red blood cells for passive haemagglutination by K2a 2c antibodies This capacity as well as *its precipitinogenic ability was practically unaffected by boiling* for 2 hours although the model strain is classified as a thermolabile L antigen strain The antibody response in rabbits against the K2a 2c antigen was studied *inter alia* by passive haemagglutination A rapid response with passive haemagglutination titres up to 1/5000 was observed after a single iv injection of *E coli* O6 K2a 2c H1 bacteria

Many *of the antigenic* factors in the model strain were also found in *E coli* O? K1 H4 and O14 K7 H By absorption of anti O6 K2a 2c H1 with bacterial extracts of these strains and with the heat stable antigen factors of the model strain an antiserum was prepared which was monospecific for an antigen presumably the H1 antigen which by comparative double diffusion was identified in the complex antigenic pattern of the model strain

Karl Axel Karlsson S Dahlstrand E Hanko & O Soderlind Research Institute of National Defence Sundbyberg National Bacteriological Laboratory and National Veterinary Institute Stockholm ON THE USEFULNESS OF THE FA TECHNIQUE IN THE DIAGNOSIS OF TULARAEMIA

In connection with an epidemic outbreak of tularaemia in 1967 ?? sylvatic animals predominantly hares have been investigated regarding tularaemia In all cases the FA technique was applied besides histopathological and/or conventional bacteriological investigations

In 124 cases all three methods were used Complete agreement was obtained in 85 cases (30 positive 55 negative) In 34 cases the results of the *histopathological* and FA investigation were positive whereas the bacteriological investigations were negative 4 cases were positive with the FA technique but could not be confirmed with the other *methods*

In one case the FA technique gave a negative *result but the other two* diagnostic procedures were positive

In 1? animals the FA technique was compared with histopathological procedures only The *results were in agreement in* 103 cases (57 positive 46 negative) The remaining cases were positive histopatholog*ically* but negative with FA technique

In 146 guinea pigs which had been inoculated with material *from cases* suspected of tularaemia infection the results of cultural and FA procedures were *compared* Both methods gave the same diagnosis in 136 cases In 9 cases the FA technique gave a positive result whereas *the cultivation* was negative In one case the FA test was negative whereas the cultivation method was positive

In experimentally infected rabbits tularaemia bacteria could *be detected* in the liver even after storage at room temperature for 2? days post mortem

According to this study the *results of the* FA technique for the detection of tularaemia in field material are in good *agreement* with the results of the time consuming histopathological examination and superior to the conventional bacteriological investigation

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F Hammarström & Klara Thiringer Department of Clinical Bacteriology The Sundswall Hospital Sundsvall THE INFLUENCE OF TEMPERATURE ON THE TRANSPORT OF MENINGOCOCCAL, GONOCOCCAL AND URINE SPECIMENS

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Universitetet i Oslo Institutt for Patologisk Anatomi
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DEGENERATIVE CHANGES IN THE PROXIMAL RENAL TUBULES FOLLOWING ADMINISTRATION OF BACITRACIN

An Ultrastructural Study in the Mouse

By

ASMUND KJERHEIM and ODD E HANSSEN

Received 23 xii 68

Plasma proteins are normally filtered through the glomeruli of the kidneys and are subsequently reabsorbed by the epithelial cells of the proximal tubules Accordingly proteinuria can be caused by three different mechanisms First protein may be excreted in the urine if the quantity of filtered protein surpasses the reabsorptive capacity of the tubular cells This may occur if the concentration of normal or pathological proteins in the blood plasma is increased or secondly if increased amounts of plasma proteins are filtered because of abnormal permeability of the glomeruli

The first of these mechanisms has been demonstrated experimentally by way of intravascular infusion of different proteins Among others *Ericsson* (3) and *Miller* (14) used homologous haemoglobin and studied the tubular reabsorption of this protein with the electron microscope Proteinuria due to the second mechanism i e increased permeability of the glomeruli occurs in clinical nephrosis in man and in nephrosis induced by aminonucleoside in experimental animals In these conditions the most striking change within the glomeruli was fusion of the epithelial foot processes Using ferritin as a tracer it was demonstrated ultrastructurally that the permeability of the glomeruli is increased in aminonucleoside nephrosis (4) These authors claimed that the primary lesion is within the basement membrane and that fusion of foot processes occurs secondarily

In both types of proteinuria vacuoles and round dense aggregates are observed electron microscopically in the tubular cells Most of these dense structures are thought to represent reabsorbed protein and probably correspond to the hyaline droplets observed by light microscopy in different types of proteinuria

Thirdly proteinuria will follow when the reabsorptive capacity of the tubular epithelium is decreased However there are few if any

clear cut examples of this category in which the glomerular function is undoubtedly normal In the present paper which is the first comprehensive report on the fine structural lesions within the proximal tubules following bacitracin we suggest bacitracin intoxication as an experimental model of this type This suggestion is based on the observations made in this study and is supported by previous light microscopical demonstrations of well maintained glomerular filtration (7) and reduced tubular reabsorption of Evans blue (6) in this condition

MATERIALS AND METHODS

Twenty one female white mice in groups of 7 were given a single intraperitoneal injection of 15 000 I U of bacitracin (A/S Apothekernes Laboratorium for Special preparater Oslo) per kg body weight The animals were killed with ether after 3 6 and 18 hrs Thin slices of kidney tissue were immediately immersed in chilled 1 per cent osmium tetroxide in veronal acetate buffer at pH 7 4 for 2 hrs dehydrated in acetone and embedded in Vestopal W Alternating thick sections for light microscopical orientation and ultrathin sections for electron microscopy were cut on an LKB Ultrotome I The sections for light microscopy were stained with a solution of 0 1 per cent toluidine blue in phosphate buffer at pH 9 0 for 2–3 min the ultrathin sections were stained with lead according to Karnovsky s method B (12) A Siemens Elmiskop I electron microscope equipped with 50 microns platinum objective apertures and double condensor illumination was used for the electron microscopy

RESULTS

Light Microscopy

The major portion of the lumens of the proximal tubules were patent and degenerative changes in the form of dark droplets or bodies were observed within the tubular epithelium Protein casts were found in the lumen particularly in the distal and collecting tubules The glomeruli were essentially normal

The pathological lesions were present after all time intervals studied but were more pronounced in the animals sacrificed 18 hr after the administration of bacitracin As the lesions were qualitatively identical the results are taken together in the following

Electron Microscopy

The fine structure of the glomeruli did not deviate from the normal as illustrated in the survey electron micrograph (Fig 1) The endothelial cells possessed fenestrations in normal amounts (Fig 2) The basement membrane was not thickened and the spaces between the foot processes were within the normal range of 200–700 Å (18)

The main pathological changes occurred within the proximal tubular cells and were observed in (1) the apical portion of the cytoplasm (2) the dense bodies (3) the endoplasmic reticulum and (4) the mitochondria



Figs 1–2

Fig 1 Survey picture of several cells from the renal glomerulus of a mouse 18 hr after a single injection of bacitracin No pathological changes are visible × 7 000

Fig 2 Portion of glomerulus from bacitracin treated mouse (18 hr) The slit pores between the epithelial foot processes are of normal size There is no thickening of the basement membrane Normal number of fenestrations in the endothelial cells × 15 000

(1) *The apical cytoplasm* This region was narrower than normal and contained vesicles of varying sizes (Fig 3) Vacuoles containing a somewhat denser material as seen in normal tubular epithelium were not observed after administration of bacitracin The microvilli of the brush border were less closely packed than normal (Fig 4)

(2) *Dense bodies* One of the most prominent changes in bacitracin intoxication was the presence of numerous membrane bounded dense bodies with heterogeneous contents (Figs 3 4 5 and 9) The majority of these bodies were larger than the dense bodies usually observed in other cells and measured 0 5–1 micron They contained parallel stacks of electron dense lamellae a dark homogeneous material resembling lipid and finely granular masses of varying density In some places remnants of cytoplasmic structures such as mitochondria and ribosomes were observed within the dense bodies The large dense bodies were observed throughout the cell and were not as in normal cells restricted to the apical area

(3) *Agranular endoplasmic reticulum* This organelle was observed as clusters of closely packed smooth vesicular and tubular elements measuring 200–700 Å in diameter (Fig 6) In other areas of the cytoplasm membrane pairs were observed surrounding mitochondria (Fig 7) or round homogeneous structures resembling microbodies (Fig 6) The paired membranes formed spirals which were often continuous with small tubules (diameter 200 300 Å) lying at the inner or the outer surfaces of the spirals (Fig 7)

(4) *Mitochondria* In most blocks the mitochondria were adequately fixed i e they were not swollen and their inner membranes were well preserved (Figs 8 and 9) Several mitochondria contained dense intramatrical granules 500–800 Å in diameter i e larger than those occurring in normal mitochondria of the tubular cells The outline of the granules was irregular sometimes polycyclic with a central less electron dense area

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Figs 3–4

Fig 3 Tubular epithelium 18 hr after administration of bacitracin The apical portion of the cytoplasm is narrower than in untreated animals A large dense body (Db) with heterogeneous membrane bounded contents is visible within the narrow tubular cell × [illegible]

Fig 4 Tubular epithelial cells following treatment with bacitracin for 18 hr The lumen is patent The microvilli are irregular and some of them have been detached Numerous dense bodies (Db) are visible throughout the cytoplasm × 16 000


Db
3


Db

L
5



DISCUSSION

The observation of normal glomeruli in bacitracin treated mice is in good agreement with previous morphological and physiological data to the effect that the glomerular filtration is well maintained after administration of bacitracin (6 7)

The observation of patent tubular lumens in bacitracin intoxication sheds some light on the problem of agonal and early postmortal processes leading to changes in the tubular lumen In normally functioning kidneys the lumen is considered to be patent (3 8) In kidneys fixed by immersion however the tubular lumens are almost invariably collapsed The reason for this seems to be that the tubular urine is reabsorbed post mortem On the other hand the tubular reabsorption of urine and subsequent collapse of the lumen can be prevented by mannitol due to the osmotic activity of this substance (8) Similarly the presence of patent lumens in bacitracin intoxication may be caused by increased osmotic activity of proteins in the tubular urine since the reabsorption of proteins is decreased in this condition

The narrow apical zone containing numerous vesicles of varying size has the same appearance as in kidneys fixed in vivo (9) This zone also has some features in common with the appearance after osmotic diuresis induced by mannitol Here again the increased osmotic activity of the protein rich tubular urine in bacitracin intoxication induces a picture resembling the in vivo situation By contrast normal tubules fixed by immersion fixation exhibit a considerably broader apical zone with vacuoles containing dense material (14)

The presence of large amounts of dense membrane bounded structures resembling digestive vacuoles and autophagic vacuoles was a typical finding in the majority of proximal tubular cells in this study Ericsson (3) and Miller (14) investigated the ultrastructural changes in the tubular cells following glomerular filtration of intravascularly injected haemoglobin They followed the tubular absorption of haemoglobin and its transformation within vacuoles to structures morphologically identical with those observed in this study Since the reabsorption of protein was decreased in bacitracin intoxication (6) the material within the membrane bounded dense bodies probably origi

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Figs 5–6

Fig 5 Dense body within a tubular epithelial cell from a bacitracin treated mouse. The body consists of myelin figures a dense homogeneous substance resembling lipid (L) and finely granular masses × 5[illegible] 000

Fig 6 Accumulation of agranular endoplasmic reticulum (Er) in a tubular epithelial cell following treatment with bacitracin for 18 hr Numerous tubules and vesicles 200 300 Å in diameter are present within a rather narrow area To the right two membrane limited structures consisting of a relatively electron dense finely granular substance are visible (microbodies Mb) × 30 000

nules from the cytoplasm rather than from the tubular lumen The major portion of large dense bodies probably *reflects cell degeneration caused by the toxic influence of bacitracin* and they should therefore be con*sidered to be autophagic vacuoles However the possibility* remains that at least some of the *dense bodies may* contain bacitracin taken up *by the cell* either from the *blood stream* or from the tubular lumen during the initial phase of intoxication thus being digestive vacuoles (1 2)

The lesions observed *in the endoplasmic reticulum of the tubular* epithelial cells were almost identical with the smooth tubules and lamellar spirals seen *in hepatocytes* after treatment with α naphtol isothiocyanate Similar but less conspicuous alterations have also been demonstrated in the liver following *administration of 3 Me DAB* (17) *Herman & Fitzgerald* (10) *found myelin figures and the formation of whorls and* agranular endoplasmic reticulum in the pancreas following treatment with ethionine and the same authors observed similar *changes in the liver as well after ethionine treatment* (11) Since changes in the endoplasmic reticulum may occur *in different organs following various types of noxious agents it seems reasonable to* presume that these alterations are *unspecific and express a general* response to injury

The *most striking* changes in the mitochondria were ring shaped or dense intramatrical structures suggesting that an electron dense sub*stance may have precipitated on a preformed* less dense *core* Similar structures within cardiac mitochondria in magnesium deficient rats have been reported by *Mishra & Herman* (15) These authors presumed that the deposits consisted of calcium Intramatrical granules of this type have also been described in normal muscle mitochondria in verte*brates (13) Peachey (16) instilled barium or strontium ions* into the toad urinary bladder and observed the development of large mitochondrial granules in the epithelial as well as the muscle cells of the bladder These granules were taken to indicate a disturbance of the water and elertrolyte balance Granules of the same appearance have *been produced by incubation of* isolated rat liver mitochondria in media containing calcium ions and inorganic phosphate (5) On the basis of these observations it seems reasonable to consider the intra

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Figs 7–8

Fig 7 Pathologically altered smooth surfaced endoplasmic reticulum *Spirals of* paired membranes surround a mitochondrion At the inner and outer surfaces of the spiral the paired membranes are continuous with tubules 200–300 Å in diameter Bacitracin for 3 hr × 40 000

Fig 8 Altered mitochondria in a tubular cell from a bacitracin treated mouse (6 hr) The intramatrical granules *often possess* a central less dense core The edge of the granules is irregular often polycyclic or scalloped *The* granules measure up to 1000 Å in diameter × 55 500







Fig 9

Portion of the cytoplasm of a tubular epithelial cell treated with bacitracin for 6 hr A major part of the mitochondria contain irregular electron dense granule with a less dense core In this electron micrograph five dense bodies (Db) with heterogeneous contents are visible × 22 000

matrical granules as deposits of calcium or magnesium probably as phosphates formed as a result of altered salt and water metabolism within the tubular epithelium

In conclusion our ultrastructural observations support the concept that the primary lesion in bacitracin intoxication is restricted to the proximal tubular cells and that the proteinuria occurring in this condition is caused by decreased tubular reabsorption of protein

SUMMARY

Single injections of bacitracin 3 6 and 18 hr prior to sacrifice produced renal lesions restricted to the proximal tubules The alterations observed were a reduced thickness of the apical portion of the tubular epithelial cell increased amounts of large dense bodies accumulations of smooth surfaced endoplasmic reticulum and membraneous whorls and numerous large intramatrical dense granules within the mito chondria

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THYMIC DYSPLASIA WITH IMMUNOLOGICAL DEFICIENCY

Report of Two Unusual Cases

By

JON LAMVIK and PETER JOHAN MOE

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The characteristic clinical features of the Swiss type of hypogamma globulinaemia are reduced numbers of lymphocytes in the peripheral blood reduced amounts of serum immunoglobulins and increased susceptibility towards infections (*Glanzmann & Riniker* 1950 *Tobler & Cottier* 1958 *Hitzig et al* 1958) The patients usually develop signs of immunological deficiency in the first months of life Recurrent infections particularly in the respiratory tract dominate the clinical course and the patients usually die before 1½ years of age The char acteristic pathological findings at post mortem are hypoplasia *of the* thymus with absence of the normal cortico medullary differentiation and a marked depletion of thymocytes In addition there is a marked cellular depletion in the white pulp of the spleen and in the lymph nodes (*Good et al* 1964)

At the Gade Institute post mortem examination has recently been performed on two unrelated infants both having dysplasia of the thymus and marked reduction of the splenic white pulp *and the lymph* nodes One of the patients had granulocytopenia thrombocytopenia and a haemolytic process At post mortem a *hypocellular* bone marrow was found The other patient had a histologically malignant lympho reticular tumour in the *spleen*

CASE REPORT

Case 1

A.J H a prematurely born male infant birth weight 1890 g and length 43 *cm.* The parents were unrelated the *mother was* 28 years old Gravida I Para I Preg nancy was uneventful until 8 weeks before term when the mother had vaginal bleedings The infant was born three weeks *later* and transferred to the Children s Hospital shortly after delivery *because of* prematurity Physical examination on admission revealed a one hour old infant having no distress and with no signs of infection Capillary haemoglobin was 18 7–18 4 *g per 100* ml leucocyte count 800 *per µl* with 8 per cent granulocytes and with 340 nucleated *red cells* per 100 leucocytes

On the fourth day *of life the infant* was hoarse but there was no signs of pneumonia He had, however on the following day shortly after feeding an attack of apnoea with convulsion and respiratory *distress* Pulmonary rales were heard

and antibiotics were given Haemoglobin was 13 4 g per 100 ml leucocytes 800 per µl and platelets 18 000 per µl Later on he had several episodes of respiratory and cardiac arrest and died 3¼ hours after the first signs of respiratory distress

Autopsy At post mortem examination performed 5 hours after death a premature infant was found without external signs of malformation

In the trachea and right bronchial tree semisolid aspirated material was present The lungs showed atelectasis with patchy haemorrhages and with a more solid consistence in the right upper lobe

The thymus was very small weight 1 5 g compared to a normal weight of 9 g The organ was pale coloured and had a myxomatous consistency

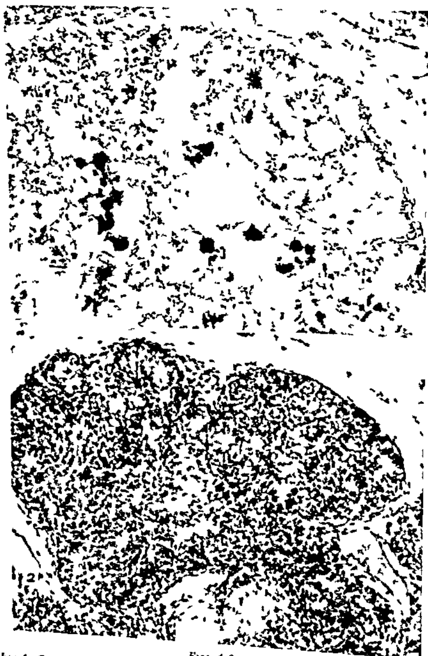
The other organs showed no macroscopic abnormality The spleen (5 g) was of normal weight No lymph nodes were found at the gross examination

The following changes were observed microscopically In the lungs acidophilic exudate was present in the terminal bronchioli and in the alveoli In addition masses of bacteria (Fig 1) which showed a positive reaction with Gram s stain were found There was practically no infiltration of inflammatory cells apart from some macrophages and nonidentified mononuclear cells in the connective tissue septa Plasma cells were not seen No inflammatory foci and no signs of bacterial invasion were found in the other organs

The thymus (Fig 2) had a lobular architecture with pale cells with an appearance like epithelial cells and reticulum cells The cells were in some areas arranged in pseudoacini No Hassal s corpuscles were found Scattered between the epithelial cells some cells with acidophilic granular cytoplasm were found The cells gave a weak PAS positive staining reaction and showed metachromasia after staining with toluidine blue Similar cells were also found in small numbers in the lymph nodes No differentiation in cortex and medulla was present and very few cells with an appearance like small lymphocytes were found between the epithelial cells

The spleen showed a normal red pulp but only a few rudimentary Malpighian corpuscles were present (Fig 3) In the mesenteric connective tissue lymph nodes with a reticular stroma were found but with a very sparse lymphocyte population The liver had a normal lobular architecture A few scattered haematopoietic foci were found in the liver sinusoids The costal bone marrow (Fig 4) was hypocellular with poorly defined leucopoiesis Megakaryocytes were fairly numerous The tunica propria and the submucosa of the small intestine contained some lymphocytic aggregates but lacking of reactive changes No abnormality was found in the other visceral organs

No abnormality was found on immuno electrophoresis of the parents sera The mother had recently born a girl infant without signs of disease



Figs 1–2

Fig 1 Case 1 Massive bacterial invasion in the lung without inflammatory response H+E, × 150

Fig 2 Case 1 Thymus with lobules of medullary tissue without Hassal's corpuscles and with very few thymocytes H+E, × 150

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Figs 3–4

Fig 3 Case 1 Spleen with a small and cell poor Malpighian corpuscle H+E × 3 0
Fig 4 Case 1 Costal bone marrow Hypocellular picture with poorly defined haematopoiesis Megakaryocytes fairly numerous H+E × 370

Case 2

K.M a male infant who died at the age of 7½ months His parents who were second cousins and 3 siblings were all healthy A sister died at the age of 2¼ months in the Children s Hospital from Staphylococcal septicaemia probably precipitated by urinary tract infection She had no lymphopenia and autopsy revealed no evidence of thymic dysplasia

The male infant had recurrent episodes of aphtous stomatitis from the age of 3 months and received several courses of antibiotic therapy At the age of 5 months he was admitted to the Children s Hospital. The stomatitis had cleared up but he developed signs of a gastro enteritis shortly after admission One month later while still in hospital he developed a pneumonia and the spleen became palpable The lungs had just before been normal roentgenologically He was treated with different types of antibiotics without effect Oral thrush was demonstrated and candida albicans cultured from the stools and bronchial secretions Mycostatin had only temporary effect on his oral thrush Terminally he had extensive pulmonary involvement considered to be due to moniliasis He died from respiratory failure

Leucocyte count ranged between 4 200 and 13 800 and the percentage of lymphocytes from 30 to 6 Studies performed 4 days prior to death revealed total protein of 5 5 g per 100 ml Paper electrophoresis showed no peak in the gammaglobulin region

Immuno electrophoresis examination of the serum from the parents and two brothers disclosed no deficiencies in the immunoglobulins

Autopsy At post mortem examination the lungs were found to be heavy with a solid consistence weighing three times the normal weight Grayish infiltrates were found on the cut sections The thymus (weight 3 g) was small with a lobular appearance The spleen weight was 70 g four times the normal weight The consistence was more solid than usual In the central part of the spleen a solid grayish tumour with a diameter of about 20 mm was found No lymph nodes were found on gross examination

On microscopical examination the lungs showed in all lobes a complex picture with atelectasis and emphysema The alveoli were filled with a proteinaceous material with vacuoles like those found in pneumocystis carinii (Fig. 5) In addition giant cells with cytoplasmatic and some nuclear inclusions were found Granulocytic infiltrates were present but few lymphoid cells and no plasma cells The alveolar membranes and the walls of the terminal bronchioli were lined by hyaline membranes The bronchial epithelium showed extensive epidermoid metaplasia

In the thymus no cortico medullary differentiation was found Medullary lobules composed of stromal cells and epithelial cells were surrounded by fatty tissue (Fig 6) Very few thymocytes were present and no Hassal s corpuscles were seen There was some interstitial fibrosis and some acidophilic granular cells which gave a strongly positive PAS reaction were found in the interstitial tissue as well as in the medullary lobules

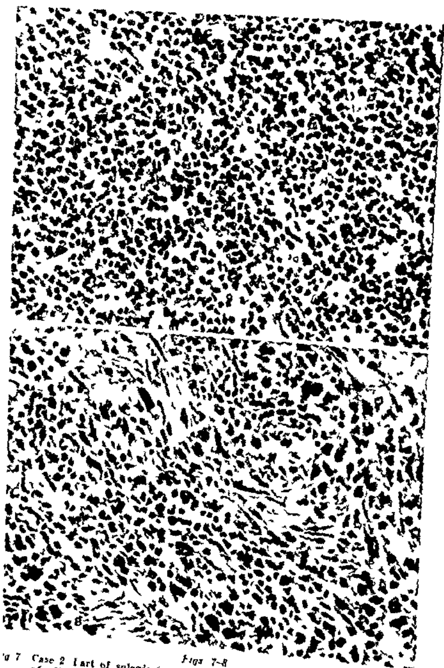
The spleen showed a prominent reticulum Lymphocytes were fairly numerous in the white pulp No germinal centres were present The centrally placed tumour was composed of lymphoid cells which in some areas had a quite uniform appearance like medium sized lymphocytes and with a general structure like a lymphosarcoma (Fig 7) In



Figs 5–6

Fig 5 Case 1 Lung with foamy material in the alveoli and moderate chronic interstitial inflammation H+E × 150

Fig 6 Case 2 Thymus with medullary lobules of stromal cells without Hassal's corpuscles The lobules are surrounded by fatty tissue without any cortical layer containing thymocytes H+E × 150



Figs 7–8

Fig 7 Case 2 Part of splenic tumour with quite uniform cell picture composed of cells like medium sized lymphocytes H+E × 370

Fig 8 Case 2 Part of splenic tumour with pleomorphic cell picture showing some atypical giant cells H+E × 370

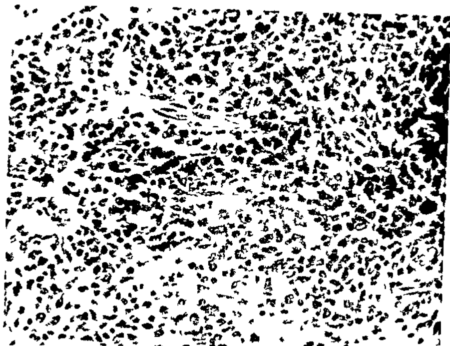


Fig 9

Case 2 Part of splenic tumour with numerous plasma cells
Methyl green – pyronin × 370

other areas the tumour showed marked variation in nuclear size and chromatin content (Fig 8) A number of giant cells were present some binucleated with prominent nucleoli In some places fibroblast proliferation with fibrosis was conspicuous In large areas of the tumour numerous cells with eccentric nuclei and strongly pyroninophilic cytoplasm were found (Fig 9) Most appeared like mature plasma cells but some were larger with immature nuclei In the central part of the tumour necrosis with calcification was present

No lymph nodes were found in the mesentery or the mediastinal connective tissue The bone marrow appeared to have a normal cellularity In the stomach duodenum and ileum very few lymphoid cells were seen and no Payer s patches noticed

No definite abnormality was found in the kidneys adrenals pancreas or thyroid Fatty degeneration was found in the liver Some small areas of liver cell necrosis were found especially bordering the portal tracts Some liver cells showed intranuclear inclusions appearing like bird eyes

DISCUSSION

The cases reported showed the characteristic pathological features of the Swiss type of hypogammaglobulinaemia dysplasia of the thymus and lack of proper development of the peripheral lymphoid organs

i e the lymph nodes and the white pulp in the spleen Both patients showed insufficient resistance against infections which caused the death in both This lack of resistance towards infections which is noticed in patients with thymic dysplasia is probably partly due to the reduced number and functional insufficiency of lymphocytes in lymphoid organs and circulating blood and partly due to deficient production of immunoglobulins Our first patient being a newborn infant was probably still supplied with immunoglobulins from the mother transferred through the placenta The second patient at 7 months of age showed a markedly reduced amount of serum gamma globulins determined by paper electrophoresis in spite of protracted infections

The first patient showed virtually no sign of cellular inflammatory response towards an overwhelming bacterial infection in the lungs This lack of cellular response is most likely explained by the leucopenia with granulocytopenia which was noticed in the blood smear on the second day of life at a time when no clinical signs of infection were present Granulocytopenia and the thrombocytopenia and *haemolytic* anaemia which also developed are not usually seen in the Swiss type of hypogammaglobulinaemia although several patients with severe immunological deficiency syndromes have developed bone marrow failure and at least one developed haemolytic anaemia *and thrombo*cytopenia (*Rosen et al* 1966) In most cases the haematopoietic deficiency developed in a late stage at a time when the patients were already suffering from severe infections (*Glanzmann & Riniker* 1950 *Good et al* 1964 *Fireman et al* 1966) Therefore the infections might have been the direct cause of the bone marrow failure

In our patient an infectious cause for the granulocytopenia appears to be unlikely If an infection had been the primary cause of the marrow failure one would expect a *cellular response* to occur at the start of infection A cellular inflammatory *picture should* be present at post mortem in some areas of the lungs or in other organs However no cellular invasion was *found* apart from a few macrophages and other large mononuclear cells in the *septa of the* lungs Furthermore granulocytopenia was present soon *after birth* at a time when no signs of disease were noticed It appears therefore that the granulocytopenia had been the *predisposing cause* for the fatal pulmonary infection The terminal drop in *haemoglobin* and platelet count may however well be secondary to the infection

The post mortem findings in our first case were very similar to those reported by *Gitlin et al* (1964) in an infant who died at 15 days of age because of an overwhelming bacterial infection without inflammatory response A similar disorder *but* with complete absence of blood leucocytes in two male newborn twins was described by *de Vaal & Seynhaeve* (1959) This disorder was *termed* reticular dysgenesia

and the authors suggested that the thymic dysplasia may represent an incomplete form of this disorder

A graft versus host reaction induced by maternal lymphocytes passing over into the foetal circulation in the prenatal period appears to be a possibility in our first patient as well as in the case reported by *Gillin et al* (1964) The mothers in both cases had uterine haemorrhage in the last two three weeks before the delivery which may facilitate a transfer of blood cells from the maternal to the foetal circulation A similar graft versus host reaction was suggested by *Hathaway et al* (1965) as the cause of a fatal pancytopenia following transfusions with viable leucocytes to two probably immunologically deficient infants The transfer of viable cells may only be harmful in immunologically deficient infants in parallel with the graft versus host reaction induced experimentally in immunologically deficient animals (*Porter* 1960 *Billingham et al* 1962 *Simonsen* 1962)

The second patient died following a clinical course which was quite typical of the Swiss type of hypogammaglobulinaemia The lack of cortico medullary differentiation and Hassal s corpuscles in the thymus and the lack of detectable lymph nodes and lymphoid tissue in the intestines are also characteristic of the disease No abnormality was found in the central nervous system

The most remarkable post mortem finding in this case was the presence of a grayish white tumour in the central part of the spleen surrounded by dark coloured pulp without obvious abnormality on gross examination The tumour tissue was composed of closely packed lymphoid cells The majority were larger than small lymphocytes with more irregular nuclei and more abundant cytoplasm In some areas definite variation in cell and nuclear size and chromatin content was noticed suggesting a malignant lymphoma In some parts of the tumour numerous cells with strongly pyroninophilic cytoplasm were found The majority were like mature plasma cells while some had immature nuclei and some were binucleated Lymphoid cell aggregates and plasma cells were not found in any other organs Thus the post mortem findings gave evidence of a localized malignant lymphoma with immature and mature plasma cells in the spleen of a patient suffering from thymic dysplasia with hypogammaglobulinaemia

Malignant lymphomas have been found to be associated with some immunologically deficiency states more commonly than would be expected on the basis of chance alone *Page et al* (1963) and *Reisman et al* (1963) have reported the occurrence of malignant lymphoma and leukaemia in three patients with congenital agammaglobulinaemia Lymphoreticular malignant tumours have been reported several times in patients suffering from ataxia teleangiectasia (*Boder & Sedgwich* 1958 *Peterson et al* 1964) This disorder is characterized by defects in the central nervous system a low serum content of IgA and increased susceptibility towards infections The thymus often shows

an abnormality similar to that described in the Swiss type of hypo gammaglobulinaemia

In the Swiss type of thymic dysplasia with hypogammaglobulinaemia malignant tumours have hitherto not been reported However because of the association between other forms of immunological deficiency states and malignant lymphomas it is not surprising that a lymphoma has also been found in this disorder *Schwartz et al* (1966) have recently reported a high incidence of malignant neoplasia in the lymphoid organs of hybrid mice with chronic runt disease after injection of parental lymphoid cells This observation may be an experimental equivalent to the malignant lymphomas seen in patients with immune deficiency states

The presence of numerous plasma cells in the tumour suggestive of a plasmocytoma is most remarkable Patients with the Swiss of hypo gammaglobulinaemia usually lack plasma cells However *Nezelof et al* (1964) and *Fireman et al* (1966) have reported two cases of thymic alymphoplasia with plasma cells in the lymph nodes and with the ability to produce one or several types of immunoglobulin Experimental studies in rats and rabbits have also given evidence of plasma cell differentiation in thymectomized animals *Waksman et al* (1962) showed that rats thymectomized at birth had normal numbers of plasma cells and normal concentration of gammaglobulin Yet the animals showed depleted lymphoid tissues and failed to make antibodies after injection of bovine serum albumin (*Jankovic et al* 1962) In the rabbit absence of the thymus may lead to plasmocytosis auto immune phenomena and amyloidosis (*Sutherland et al* 1965) This finding may be relevant to the plasmocytosis observed in the splenic tumour The patient showed however no reactive plasmocytosis in the lungs which were infected with organisms which usually give a marked degree of plasmocytosis Thus the aggregation of plasma cells in the tumour appears to be an expression of neoplastic cell proliferation and not a cellular reaction induced by antigens

SUMMARY

Two infants with thymic dysplasia and poor resistance towards infections are reported One infant developed granulocytopenia thrombocytopenia and haemolytic anaemia shortly after birth No inflammatory reaction was found in the lungs where a marked degree of bacterial invasion was found The other infant suffered from protracted gastro intestinal and pulmonary fungus infections and died in respiratory failure At post mortem a malignant lymphoma with numerous plasma cells was found in the spleen No plasma cell reaction was present in the lungs which showed a chronic inflammatory reaction

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HYALINE MICROTHROMBI IN AN AUTOPSY MATERIAL

A Quantitative Study with Discussion of the Relationship to Small Vessel Thrombosis

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Hyaline microthrombi (HMT) (22 23 24 25) are globular eosinophilic structures which are found in the microcirculation of many organs usually unattached to the vessel wall (Fig 1) HMT have a diameter which varies between 2–3 μ and 30 40 μ They give positive staining reactions for fibrin Such structures were first reported by *Welti* (26) *Apitz* (3 4) stated that they were formed by aggregation (Koazerva tion) of fibrin as a result of pathological coagulation He claimed that this usually took place after death and therefore suggested that HMT were of no significance *Zink* (27) on the other hand considered them to be a special form of thrombi formed *in vivo* in streaming blood

Skjørten (25) restudied the nature of HMT Immunohistochemically they seemed to consist of fibrinogen or fibrin Ultrastructurally however they differed from ordinary fibrin thrombi by being composed of an irregular mosaic of fine filaments which only occasionally showed the periodicity of fibrin

In routine histological sections HMT are not easily seen We first observed them in fibrin stained sections from patients with disseminated intravascular coagulation (DIC) and proposed that they might be the primordial body in such conditions (23) Subsequently however we have founnd HMT in tissues from many patients without DIC Therefore it became necessary to undertake a quantitative study of the occurrence of HMT in a consecutive autopsy series The results are reported here and are compared with the results of a quantitative study of HMT in a series of cases showing various manifestations of the generalized Shwartzman reaction

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MATERIAL AND METHODS

This report is based on the study of 100 consecutive autopsies performed in the Department of Pathology Ullevaal Hospital (autopsy no 1–100/58) Case histories and autopsy reports were unknown to the author when the microscopical studies were carried out Furthermore six cases of generalized Shwartzman reaction previously reported (24) were restudied with special reference to the occurrence of HMT

Sections from all available tissue blocks were recut and subjected to microscopical examination The number of cases with sections available from the pituitary liver kidneys and lungs is indicated in Table 1 Blocks from only 12 brains were available brains were therefore excluded from the study

Autopsies had been carried out 12–58 hours post mortem Tissues were fixed in 4 per cent unbuffered formaldehyde solution and embedded in paraffin Sections were cut at 5–10 μ and stained with haematoxylin and eosin (H+E) phosphotungstic acid haematoxylin (PTAH) (16) and Martius scarlet blue (MSB) (15)

All MSB sections were systematically screened at 300 times magnification using a Reichert Zetopan Microscope with a calibrated cross table Every HMT fulfilling the diagnostic criteria stated above and having a diameter larger than an erythrocyte were counted in each section In the pituitary particular care was taken not to confuse HMT with proteinaceous material lying centrally in glandular acini because these structures frequently showed red colour in MSB stained sections similar to fibrin Only those HMT which with certainty were lying within vessels were counted

In the following conventional mural or occlusive thrombi composed of platelets fibrin or both will be called *thrombi* The occurrence of thrombi in small vessels arterioles capillaries and venules as well as the presence of precipitated *fibrin in* tissues was studied in PTAH sections No attempt was made to quantitate thrombi or tissue fibrin but their presence or absence was recorded

The exact outline of each section was drawn on thin cardboard cut out with a pair of fine scissors and weighed on a Mettler H 4 scale (F Mettler Zurich Switzerland) to determine the area studied in each section *The number of HMT* per cm² section area was then computed for each organ in all cases studied and will subsequently be called *density*

When the quantitative evaluation was finished all case histories and autopsy reports were studied and pertinent information regarding past and present illnesses as well as autopsy findings were recorded

The results were subjected to statistical analysis computing χ^2 with Yates modification or the Fisher exact probability test in 2 × 2 contingency tables (17) the Students t test (6) and the coefficient of association (24) All P values below the 5 per cent level were considered *significant* Two tailed tests were found suitable in all situations studied

RESULTS

1 *Consecutive Series*

Occurrence of Hyaline Microthrombi (HMT)

In the consecutive series HMT were found in 54 out of 100 cases (Table 1) More than half the cases showed HMT in the pituitary The liver kidneys and lungs showed a considerably much lower frequency of HMT (Tables 1 and 3) In other organs only occasional HMT were found

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Figs 1–2

Fig 1 Hyaline microthrombi (HMT) in pituitary sinusoid. PTAH 160 ×

Fig 2 Conventional thrombi in alveolar capillaries (arrows) and intraalveolar fibrin formation in bronchopneumonia PTAH 240 ×


HMT

TABLE 1

Frequency and Density of HMT in Various Organs Consecutive and Shwart man Series

Organ	Total	Frequency			Density	
		HMT cases	No HMT	P	All cases	HMT cases
Pituitary						
Consec	87	45	42	N s	30 3	58 6
Shw	5	4	1		227 0	284 0
Liver						
Consec	97	21	76	< 0 05	1 2	5 3
Shw	6	4	2		5 2	7 9
Kidneys						
Consec	97	15	82	< 0 001	0 4	2 6
Shw	6	5	1		1 6	2 0
Lungs						
Consec	95	11	84	< 0 025	0 2	1 8
Shw	5	3	2		0 4	0 8
Other						
Consec	96	3	93	< 0 0005	0 1	3 4
Shw	6	4	2		4 7	7 0
All organs						
Consec	100	54	46	< 0 10	2 3	4 3
Shw	6	6	0		8 4	8 4

Total = total number of cases with sections available P = probability of difference between occurrence of HMT in consecutive and Shwartzman series Density = number of HMT per cm² section area Consec = consecutive series Shw = Shwartzman series N s = not significant

The mean density of HMT per cm section area was 10 times higher in the pituitary than in the liver while the kidneys and lungs showed an even lower density The mean density of HMT was 2 3 for the whole series and 4 3 when cases without HMT were excluded (Table 1) 28 cases showed HMT in only one organ 13 cases in two 11 in three and two cases showed HMT in four organs

Occurrence of Small Vessel Thrombosis

Thrombi were found in small vessels—arterioles capillaries and venules— in 45 cases (Table 2) 35 of which also showed HMT Sections from the lungs were available in 95 cases Thrombi in small pulmonary vessels were found in 36 cases Among these 32 had bronchopneumonia and showed thrombi in the inflamed areas frequently at the periphery of the bronchopneumonic nodules (Fig 2) HMT were rare in this location Within the bronchopneumonic nodules there was usually marked intra alveolar fibrin formation In the consecutive series 78 per cent of the cases with bronchopneumonia showed thrombi in small pulmonary vessels

TABLE 2

Frequency of Small Vessel Thrombosis in Various Organs Consecutive and Shwartzman Series

Organ	Total	Frequency		P
		Cases with thrombi	Cases without thrombi	
Pituitary				
Conse	87	9	76	N s
Shw	5	1	4	
Liver				
Consec	97	2	95	N s
Shw	6	1	5	
Kidneys				
Consec	97	9	88	< 0 0005
Shw	6	5	1	
Lungs				
Consec	95	36	59	N s
Shw	5	1	4	
Other				
Consec	96	3	93	< 0 0005
Shw	6	6	0	
All organs				
Consec	100	45	55	< 0 05
Shw	6	6	0	

Total = total number of cases with sections available P = probability of difference between occurrence of small vessel thrombosis in consecutive and Shwartzman series Consec = consecutive series Shw = Shwartzman series N s = not significant

TABLE 3

Distribution of HMT and Small Vessel Thrombi in Various Organs Consecutive Series

	Pituitary	Liver	Kidneys	Lungs	Heart	Other	All organs
Per cent of cases showing HMT	51 7	21 6	15 5	11 6	1 1	2 8	54 0
Per cent of cases showing small vessel thrombosis	10 3	2 1	9 3	37 9	[illegible] 2	2 8	45 0

The distribution of small vessel thrombi in various organs is shown in Table 3 About 10 per cent of the pituitaries showed thrombi in sinusoids of the anterior lobe while nine per cent of the kidneys showed thrombi in arterioles glomerular capillaries and medullary veins The liver and heart showed a low frequency of small thrombi

In the consecutive series 25 cases showed small vessel thrombosis

in only one organ 14 in two organs and six cases in three organs In 27 cases small vessel thrombosis was thought to be secondary to pathological processes adjacent to the thrombosed vessels such as bronchopneumonia infarction or malignant tumours In 18 cases no such local cause of small vessel thrombosis was found These cases failed to show significantly higher frequency of bronchopneumonia than the rest of the material Furthermore the frequency of HMT was not higher in this group than in the 27 cases with small vessel thrombosis secondary to local pathological processes

Hyaline Microthrombi and other Pathological Findings

With a view to the following statistical evaluation the consecutive autopsy series was divided in cases with and without HMT The mean section area studied per case in the HMT group was 12 7 cm² and in cases without HMT 11 5 cm² The difference was not significant ($t = 1\ 29$ $0\ 20 < p < 0\ 30$) The entire material showed a preponderance of males and of patients above 60 years of age as was to be expected in an autopsy material The sex rate in the HMT cases corrected for the preponderance of males in the entire material was 1 25 1 In spite of difference in sex ratio between HMT cases and the entire material a comparison between the occurrence of various clinical and pathological findings in cases with and without HMT is considered permissible

Study of the case histories and autopsy records (Table 4) revealed that there was a strong association between HMT and microscopically verified bronchopneumonia significant at the 0 1 per cent level The association between HMT and thrombi in small vessels was even stronger however and significant at the 0 05 per cent level Cases with thrombi in small vessels showed a mean of 4 2 HMT per cm² section area while cases without thrombi in small vessels had a mean of 0 7 HMT per cm² section area This difference is significant at the 5 per cent level ($t = 1\ 96$) When cases with bronchopneumonia and malignant tumours were excluded from the material the association between HMT and small vessel thrombosis was significant at the 0 25 per cent level (Table 4) Finally when cases with small vessel thrombosis were excluded from the material bronchopneumonia showed no association with HMT (coefficient of association = 0 064)

No significant association was found between HMT and malignant tumours without bronchopneumonia When cases with bronchopneumonia were excluded there was no significant association between HMT and deep vein thrombosis arterial emboli and thromboses or atherosclerotic heart disease (Table 4) The consecutive series included only there cases of shock defined as blood pressure below 90 mm Hg systolic for more than 6 hours before death Two of these cases showed HMT and one did not

No association between the time elapsed from death to autopsy and

TABLE 4

ssociation between HMT and Various Autopsy Findings Consecutive Series

Finding	Per cent in cases with HMT	Per cent in cases without HMT	Coefficient of association	P
ll vessel thrombosis	64 8 (35/54)	21 7 (10/46)	0 738	< 0 0005
ichopneumonia	57 4 (31/54)	21 7 (10/46)	0 658	< 0 001
gnant tumours excluded				
nall vessel thrombosis	59 0 (29/49)	0 0 (0/30)	1 000	< 0 0005
ronchopneumonia	59 5 (25/42)	10 0 (3/30)	0 856	< 0 000,
ichopneumonia excluded				
nall vessel thrombosis	30 4 (7/23)	13 9 (5/36)	0 512	N s
alignant tumours	26 1 (6/23)	25 0 (9/36)	0 003	N s
ignant tum urs and nchopneumonia excluded				
mall vessel thrombosis	35 5 (6/17)	0 0 (0/27)	1 000	< 0 0025
eep vein thrombosis nd pulmonary embolism	47 1 (8/17)	29 6 (8/27)	0 363	N s
rterial thrombosis nd embolism	29 4 (5/17)	22 2 (6/27)	0 186	N s
therosclerotic heart disease	58 5 (10/17)	51 5 (14/27)	0 140	N s

Absolute numbers are given within parentheses N s = not significant

TABLE 5

Association between Small Vessel Thrombosis and Various Autopsy Findings Consecutive Series

Finding	Per cent in cases with small vessel thrombosis	Per cent in cases without small vessel thrombosis	Coefficient of association	P
onchopneumonia	73 3 (33/45)	14 5 (8/55)	0 884	< 0 0005
alignant tumours excluded				
Bronchopneumonia	79 3 (23/29)	11 6 (5/43)	0 933	< 0 0005
r nch pneumonia excluded				
Malignant tumours	58 3 (7/12)	17 0 (8/47)	0 744	< 0 025
al gnant tumours and ronchopneumonia excluded				
Deep vein thrombosis and pulmonary embolism	50 0 (3/6)	34 2 (13/38)	0 316	N s
Arterial thrombosis and embolism	33 3 (2/6)	23 7 (9/38)	0 234	N s
Atherosclerotic heart disease	50 0 (3/6)	55 2 (21/38)	—0 105	N s

Absolute numbers are given within parentheses N s = not significant

he occurrence of HMT was found The possible relationship between stasis and occurrence of HMT was studied but not subjected to quantitative analysis The finding of HMT alone or adjacent to scattered red cells was as frequent as the finding of HMT in vessels showing red cell stasis

Small Vessel Thrombosis and other Pathological Findings

Associations between thrombi in small vessels and various pathological findings are given in Table 5 Microscopically verified bronchopneumonia showed a strong association with small vessel thrombi significant at the 0 05 per cent level When bronchopneumonia was excluded from the material malignant tumours showed a positive association with thrombi in small vessels significant at the 2 5 per cent level Many of these thrombi however were located in necrotic areas within the tumour Neither deep vein thrombosis and pulmonary embolism atherosclerotic heart disease nor arterial thrombi and emboli showed any association with thrombi in small vessels when cases with bronchopneumonia and with malignant tumours had been excluded from the material

TABLE 6

Association between HMT and Small Vessel Thrombosis for Various Autopsy Findings Consecutive Series

Finding	Per cent of cases with HMT showing small vessel thrombosis	Per cent of cases without HMT showing small vessel thrombosis	Coefficient of association	P
Bronchopneumonia	90 3 (28/31)	50 0 (5/10)	0 806	< 0 025
Malignant tumours excluded				
Bronchopneumonia	92 0 (23/25)	0 0 (0/3)	1 000	0 006
Bronchopneumonia excluded				
Malignant tumours	33 3 (2/6)	55 5 (5/9)	—0 429	N s
Malignant tumours and bronchopneumonia excluded				
Deep vein thrombosis and pulmonary embolism	37 5 (3/8)	0 0 (0/8)	1 000	N s
Arterial thrombosis and embolism	40 0 (2/5)	0 0 (0/6)	1 000	N s
Atherosclerotic heart disease	30 0 (3/10)	0 0 (0/14)	1 000	N s

Fisher s exact probability test is employed in all instances except for bronchopneumonia (first group) N s = not significant

Hyaline Microthrombi and Small Vessel Thrombosis

The association between HMT and thrombi in small vessels was studied for various clinical and pathological findings (Table 6) Cases of microscopically verified bronchopneumonia showed a positive association between HMT and small vessel thrombi significant at the 2 5 per cent level When malignant tumours were excluded the association became significant at the 0 6 per cent level Malignant tumours without bronchopneumonia showed a negative but not significant association between HMT and small thrombi When bronchopneumonia and

malignant tumours were excluded there was a positive but not significant association between small vessel thrombi and HMT in cases with deep vein thrombosis and pulmonary embolism atherosclerotic heart disease and arterial thrombosis and embolism

2 Shwartzman Series

The frequency and density of HMT in the Shwartzman series are given in Table 1 All organs showed a higher frequency of HMT than in the consecutive series The difference was significant in all instances except for the pituitaries Table 2 shows that the frequency of small vessel thrombosis was higher in the Shwartzman series than in the consecutive material There was a significant difference for kidneys other organs and all organs

The mean HMT density per cm² section area showed large variation from case to case in the Shwartzman series Cases in which the interval between the last episode of disseminated intravascular coagulation (DIC) and death covered from one to four days as judged from case histories and microscopical findings had the highest HMT density (Table 7) All cases had widespread small vessel thrombosis

TABLE 7

Density of HMT and Temporal Relationship to DIC Individual Cases Shwartzman Series

Case number	1	2	3	4	5	8
Density of HMT	1 95	0 41	31 36	6 49	7 31	2 0
Interval DIC to death days	10	½	1 2	3–4	3–4	28
Number episodes of DIC	1	1	several	several	2	1

Density of HMT = number of HMT per cm² section area
Interval DIC to death = interval from last episode of DIC to death

DISCUSSION

Occurrence of Hyaline Microthrombi

HMT were found in 54 per cent of our consecutive autopsies We are unaware of any previous study of the occurrence of HMT in a consecutive material Apitz (3) stated that HMT were a rare finding our study shows that they are not

HMT were found in more than half the pituitaries in one fourth of the livers somewhat less frequently in the lungs and kidneys but rarely in other organs Furthermore the density of HMT in the liver was one tenth of that in the pituitary the kidneys showed a still lower density of HMT and the heart showed only a few HMT Blood flow per gram tissue is high in the kidneys however (9) and lower in

the liver (13) pituitary (18) and heart (7) The different density of HMT in these organs can therefore not be ascribed to differences in *blood flow*

Apitz (4) found HMT most frequently in the brain We had to exclude the few brain sections present from our quantitative study It is our impression however that HMT are considerably less common in the brain than in the pituitary

In a previous study (25) we presented evidence that HMT are formed *in vivo* In the present study there was no association between occurrence of HMT and interval between death and autopsy This finding further supports our view that HMT are formed *in vivo*

Occurrence of Small Vessel Thrombosis

In our consecutive series small vessel thrombosis was found in 45 per cent of the cases Close to 40 per cent of the lungs and about 10 per cent of the pituitaries and kidneys showed small thrombi but only two per cent of the livers and hearts *Haerem* (11) found thrombi in small arteries in about three per cent of 215 hearts *in a consecutive* autopsy material We have been unable to find other figures on the frequency of small vessel thrombosis in a consecutive autopsy material

In our material small vessels were mainly occluded by fibrin thrombi but occasional platelet thrombi were also seen *Eeles & Sevitt* (10) made similar findings in a study of small vessel thrombosis in injuries and burns

Relationship of Hyaline Microthrombi to Small Vessel Thrombosis

In our consecutive series there was a highly significant association between HMT and small vessel thrombosis In spite of this the distribution pattern of the two types of thrombi was different with maximal frequency in different organs This could mean that conditions which lead to the formation of thrombi in small vessels promote the formation of HMT by altering the composition of the plasma so that HMT are formed in streaming blood as suggested by *Zinck* (27) Subsequently HMT might tend to become caught in organs with sinusoidal blood flow or complicated vascular pattern *Zinck* (27) suggested that slow blood flow favoured the formation of HMT We tried to evaluate the relationship of stasis to HMT with negative results

In our material bronchopneumonia showed a strong positive association with HMT which however rarely were found in the lungs in these cases When cases with small vessel thrombosis were excluded from the material bronchopneumonia failed to show a higher frequency of HMT than the rest of the material Furthermore cases without bronchopneumonia also showed a significant association between HMT and small vessel thrombosis These findings are a strong indication that the formation of HMT is causally related to small vessel thrombosis and not to bronchopneumonia

In a previous paper (23) we proposed that HMT might lead to progressive thrombosis after having been embolized to small vessels of various tissues. Changes which might indicate progressive thrombosis around HMT are rare however. We have seen such changes less than ten times. *Zink* (27) discussed the possibility of progressive thrombosis around HMT but stated that he had not observed it. *Hardaway et al* (12) suggested that progressive thrombosis might occur around HMT but this phenomenon is not illustrated in their report.

The observation of HMT surrounded by conventional thrombi indicates that HMT are formed *in vivo* but not necessarily that HMT have initiated progressive thrombosis. During the formation of conventional thrombi in small vessels HMT might occasionally be formed near by and in rare instances become surrounded by conventional thrombus material.

Ashford et al (5) found high fibrinolytic activity in veins with a diameter less than 50 µ. HMT are strongly associated with small vessel thrombosis. *Skjørten* (25) demonstrated that the ultrastructural pattern of HMT differs from that of ordinary fibrin indicating a different mode of polymerization. *Alkjaersig et al* (2) have shown that fibrinogen split products interfere with the normal polymerization of fibrin. Therefore the formation of HMT might possibly be related to activation of the fibrinolytic system initiated by small vessel thrombosis.

Small Vessel Thrombosis and Bronchopneumonia

Lobar pneumonia is associated with intra-alveolar fibrin formation and extensive capillary thromboses (14, 20). We found small vessel thrombosis in 78 per cent of our cases of bronchopneumonia. Such a high frequency of capillary thrombosis in bronchopneumonia is not mentioned in most texts on the pathology of pneumonia.

In experimental DIC produced by intravenous thrombin infusion large amounts of fibrin are found in pulmonary vessels small and large (1). In this situation however there is very little or no fibrin in pulmonary alveoli. On the other hand in bronchopneumonia intra-alveolar fibrin is formed as a part of the inflammatory exudate. Capillary thrombi are found scattered in the periphery of the bronchopneumonic nodules and not in parts of the lungs which are unaffected by the inflammatory process. This indicates that in bronchopneumonia small vessel thrombosis in the lungs is related to the inflammation and is not a result of DIC or embolization. However in exceptional cases (8, 24) massive pneumonia with pneumococcal septicemia may precipitate DIC.

Hyaline Microthrombi and Small Vessel Thrombosis in other Pathological Conditions

Malignant tumours showed a significant positive association with small vessel thrombosis even when cases with bronchopneumonia were

excluded but no association with HMT *McKay* (17) reported three cases of malignant tumours with disseminated small vessel thrombosis and proposed that thrombosis might have been precipitated by the release of thromboplastin substance from the tumour itself Our consecutive series included 28 cases of malignant tumours None of these showed disseminated small vessel thrombosis In most of our cases small vessel thrombosis was found in the tumour itself in or adjacent to necrotic areas in which there appeared to be very limited circulation This might explain the lack of association between HMT and small vessel thrombosis in our tumour cases

The consecutive series included 16 cases with deep vein thrombosis and pulmonary embolism when bronchopneumonia and malignant tumours had been excluded These cases showed neither a significant association with HMT nor with small vessel thrombosis The number of cases in each of these groups is small and does not justify any conclusion regarding the relationship of HMT and small vessel thrombosis to thrombosis in large vessels

Hardaway et al (12) found HMT in small vessels in 29 out of 3? cases of shock In our consecutive series only three cases of clinical shock were recorded two of which showed HMT One of the latter also showed small vessel thrombosis In a retrospective study like ours however many cases of shock may have escaped recognition and been included in other groups Our material gives no information on the occurrence of HMT in healthy subjects dying suddenly *Remmele & Harms* (19) found HMT in only one out of 56 cases of sudden death which indicates that HMT are rare in this condition

Remmele & Harms (19) studied 112 cases of shock and found small vessel thrombosis in 61 cases with a 50 per cent frequency in livers kidneys and lungs These figures include HMT as well as ordinary thrombi Still they show a considerably higher frequency of thrombi than that observed in our consecutive series and thus support experimental and clinical studies reviewed by *McKay* (17) which indicate that shock is frequently associated with widespread small vessel thrombosis *Eeles & Sevitt* (10) studied the occurrence of small vessel thrombosis in severe injuries and burns and found a lower thrombus frequency than the one observed in our consecutive series Technical differences may account for this apparent discrepancy

Hyaline Microthrombi and Disseminated Intravascular Coagulation

In the Shwartzman series the density of HMT varied from 0 4 to 31 1 per cm section area A high density was found in cases with evidence of two or more episodes of intravascular coagulation going on until the last days before death Two cases which showed signs of only one episode of DIC having taken place 10 and 23 days before death respectively showed few HMT This might mean that the mechanisms

which facilitate the formation of HMT were no longer active and that HMT previously formed had been eliminated from the circulation On the other hand a case of meningococcal septicaemia which led to death in 12 hours had the lowest number of HMT in this series in spite of the fact that there was extensive small vessel thrombosis in glomerular capillaries adrenals and skin In this situation the mechanisms promoting the formation of HMT might still be poorly activated because of the short duration of the disease

DIC is an intermediary mechanism of disease (17) in a number of different and frequently fatal conditions in which small vessel thrombosis may vary in intensity from cases showing only a few thrombi in scattered organs to cases with a generalized Shwartzman reaction In our consecutive series 18 cases showed small vessel thrombosis which could not be ascribed to local processes adjacent to the thrombosed vessel such as inflammation and necrosis We believe that these cases may represent a low degree of DIC which therefore seems to be a fairly common event in critically ill patients The generalized Shwartzman reaction on the other hand is rare having a prevalence in our laboratory of about one per 1000 autopsies (24)

In a previous paper (23) we suggested that HMT were the first thrombi formed in DIC and that small vessel thrombosis might take place around embolized HMT The present paper has failed to support this theory We have found HMT in patients with small vessel thrombosis caused by bronchopneumonia as well as by DIC It seems that the occurrence of HMT is independent of the cause of small vessel thrombosis The presence of HMT in microscopical sections is a strong indication that small vessel thrombosis has also taken place but not necessarily in the same organ The presence of HMT in sections from several organs indicate that DIC may have taken place but is not diagnostic of this condition

SUMMARY

The occurrence of hyaline microthrombi (HMT) was studied quantitatively in 100 consecutive autopsies and in six fatal cases of generalized Shwartzman reaction HMT were found in 54 cases in the consecutive series with a mean density of 2 3 HMT per cm² section area They found in all Shwartzman cases with a mean density of 8 4 HMT per cm² section area

Small vessel thrombosis was found in 45 cases in the consecutive material 20 of these had thrombi in two or three organs Small vessel thrombosis which could not be ascribed to local processes adjacent to the thrombosed vessels were found in 18 cases HMT showed a highly significant association with small vessel thrombosis and had maximal frequency in the pituitary while small vessel thrombosis was most commonly found in the lungs in relation to bronchopneumonic nodules Small vessel thrombosis was found in 78 per cent of the cases of

bronchopneumonia studied Small vessel thrombosis appears to facilitate the formation of HMT, possibly through the simultaneous activation of the fibrinolytic system

It is concluded that the demonstration of HMT is a strong indication that small vessel thrombosis also has taken place but not necessarily in the same organ HMT occur with high frequency in cases of disseminated intravascular coagulation but are not diagnostic of this condition

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QUANTITATIVE STUDIES ON CORTISOL INDUCED DECAY OF LYMPHOID CELLS IN THE THYMOLYMPHATIC SYSTEM

By

MOGENS HELWEG CLAESSON and CARSTEN RØPKE

Received 8 i 69

Several attempts to estimate the number of pyknotic cells in the thymo lymphatic system of the normal rat and mouse have been made (4 13 18 21 23) In a recent study it was shown that a high number of dead and dying lymphoid cells could be registered through the use of Dye Exclusion Test (D I T) on unfixed lymphoid organ suspensions (4) The number of dead and dying cells registered in this manner exceeded several times the number of above mentioned pyknotic cells

In the light of this observation (4) we searched for an experimental agent which would change the ratio between normal and dying lymphocytes in the thymolymphatic system It is well known that a single injection of cortisol given to an animal or a human being will cause a rapid fall in the number of circulating lymphocytes and a strong involution of various lymphoid organs The microscopic picture of the involution shows massive pyknosis karyorrhexis and cytoplasmatic budding of the lymphocytes These changes are followed by a rapid phagocytosis of nuclear fragments The most pronounced degree of involution takes place in the thymus (5 8 18 22 25)

The aim of the present study was by application of the DIT to investigate (1) the cortisol induced involution of the thymolymphatic system (2) the ratio of the number of pyknotic cells to the number of stained cells in the thymus during treatment with cortisol (3) the use of stained thymolymphoid cell number as a kinetic parameter in the study of cortisol induced involution and regeneration of the thymus gland

MATERIAL AND METHOD

Male and female albino mice of the C strain were examined The animals were housed under standard conditions They were separated in two groups according to age and experimental procedure In the first group (25 mice 2 months of age) each animal received 5 mg In the second group (60 mice 1 month of age) 1 mg of cortisol (hydrocortisoneacetate dissolved in sterile 0 9 per cent saline solution LEO) injected intraperitoneally One hour to 14 days after the injection 4 to 10

animals from each group were examined As control several untreated animals were examined during the experimental period

Animals were killed by cervical dislocation In the first group the thymus mesenteric and axillary lymph nodes were removed and immediately transferred to chilled Hank solution In the second group only the thymus was removed Single cell suspensions from the various lymphoid organs were prepared and the nigrosin dye exclusion test performed as described in detail elsewhere (4) In the second group the thymus gland was weighed wet and one lobe transferred to Lillie s fixative and embedded in paraffin Six microns slices were sectioned in an ordinary microtome Slices stained in H E Feulgen and gallocyanine formed the basis for estimation of the degree of involution and the incidence of mitosis and pyknosis

Several technical factors are of importance with regard to the result of DET It is especially important that the dye concentration the staining period the density of the suspension and the temperature are kept within certain critical limits (2 4 9 20 24) Preliminarily a technique that ensures reproductibility of the DET was studied in lymphoid cell suspensions (3)

RESULTS

With present suspension techniques the ratio of free lymphoid cells to accidental fixed reticular cells is app 50 1 as decribed in detail elsewhere (4 21) In lymphoid organ suspensions from animals *treated* with cortisol the number of free lymphoid cells pr mg organ weight decreases sharply yet the ratio of free to fixed cells remains unchanged Fig 1 shows graphically the percentage of free nigrosin stained *cells in* relation *to the total* number of free cells in various lymphoid organ suspensions Each point represents mean percentage of *stained* cells in suspensions of thymus mesenteric or axillary *lymph nodes* from 5 to 10 animals Bars represent standard deviation *of each* sub group The abscissa indicates hours and days after injection of cortisol the ordinate indicating the percentage of stained cells

As indicated in Fig 1 a rapid increase in the *number of* stained cells from various lymphoid organs follows injection *of 5 mg* of cortisol reaching a maximum on the 5th day It shows furthermore the particular sensitivity of thymus *to cortisol* The number of stained thymus lymphoid cells increases 4½ times while the number of stained lymph node cells increases only 2 to 2½ times and *does not* reach control level at the end of the experimental period

Fig 2 shows variations in percentage of stained thymo lymphoid cells after injection of 1 *mg of cortisol* into mice 1 month of age The table includes relative thymus *weights* (*mg* thymus/g mouse) Each point represents the mean weights of all thymus glands in a subgroup In this experiment variations *during the* first 24 hours were of special interest In the first 6 hours the *number* of stained cells increases up to twice the control level At 12 hours the number reaches a minimum below the control level Then a gradual rise follows reaching a maximum on the 3rd day *On the 10th* day of examination the number of stained cells reaches control level again Also Fig 2 shows that relative thymus weight decreases to *about half* the weight of the control thymi during the first 6 hours Throughout the following 18 hours this weight

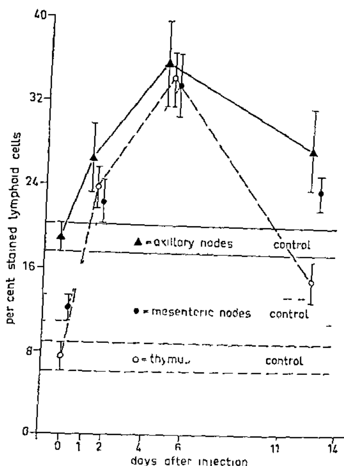


Fig 1

Cortisol induced variation in the number of nigrosin stained lymphoid cells in single cell suspensions from thymus mesenteric and axillary lymph nodes from mice injected i p with 5 mg of cortisol at time 0

remains relatively constant A decrease follows lasting from 24 hours to the 3rd day the weight reaching a minimum of app 1/4 of the weight of the control group From the 3rd day and through thymus weight increases but never reaches control level

Cell pyknosis and karyorrhexis occur particularly during the first day after cortisol injection appearing in thymus suspensions as well as in fixed thymus preparations Numerical estimations of cell pyknosis were based on fixed preparations where pyknoses are easily recognizable For the same reason estimation of mitotic frequency was performed on these fixed preparations In the 6 hour group a rise in the number of pyknoses in the thymic cortex is found The pyknoses showed diffuse distribution Also in the thymic medulla the incidence of pyknoses is slightly increased Later in the 12 hour group pyknoses are seen to gather around fixed reticular cells filled with lymphocyte nuclear frag

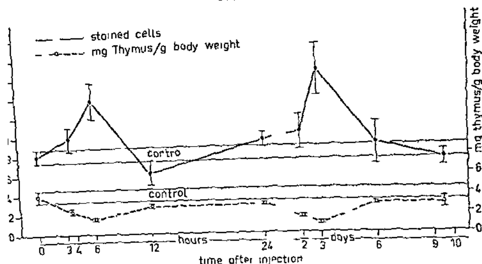


Fig 2

Change in number of nigrosin stained lymphoid cells in single cell suspensions from thymus, induced by cortisol injection Also change in relative thymus weights induced by cortisol injection

ments Still there is a tendency toward diffuse cell pyknosis in cortex and medulla In the 24 hour group pyknoses have disappeared In the cortex however a few reticular cells are visible, loaded with nuclear fragments At this time the cortex is nearly depleted of small lymphocytes

The number of mitoses during the first 12 hours does not change although many mitoses in the 6 hours group have an abnormal appearance At 24 hours mitotic frequency has decreased to a point where but a few immature cells are found to undergo mitosis On the 3rd day the number of mitotic cells increases strikingly From then on mitotic frequency remains at control level

DISCUSSION

According to studies by *Dougherty et al* (1964) and others (5 8 22 23) cortisol induced injury to lymphoid cells involves both thymus and other lymphoid organs Injury is heaviest in the thymus gland however These studies used thymus weight cell pyknosis karyorrhexis and 17 hydroxy dehydrogenase activity in various lymphoid organs as expressions of cortisol induced involution The change in these parameters reaches a maximum shortly after injection of cortisol According to the Dye Exclusion Test however lymphoid cell decay continues and increases several days after injection of cortisol see Fig 1 Apparently as in the above mentioned studies the thymic lymphoid cells are particularly sensitive to cortisol The effect of cortisol seems to persist since the number of stained cells continues and increases a

long time after disappearance of cortisol from the organism The half life of free cortisol in the blood of mouse is about 40 to 50 minutes thus by 100 minutes all the administered cortisol is removed (5)

The number of dead thymic lymphoid cells (Fig 2) registered by D E T does not follow the pyknotic frequency found in the fixed preparations Like others (5 8 22 25) we find a maximum degree of pyknosis within the first day after injection of cortisol

We believe that cell pyknosis and karyorrhexis both are expressions of an acute effect of cortisol on small lymphocytes while nigrosin staining mostly results from a protracted effect of cortisol on more primitive lymphocytes incl inhibition of DNA replication and inhibition of mitosis at the metaphase stage Thus we find no simple relationship between the number of cell pyknosis and the number of cortisol induced stained cells Rather the two kinds of transformation registered here appear to be independent expressions of cell decay *Meyers & Wolfe Slade* (1964) who studied the effect of X irradiation of thymic cell suspensions share this view

During the first 6 hours the decrease in relative thymus weight (from 4 mg to 2 3 mg see Fig 2) is an actual result of changes of which massive cell pyknosis and increased cell stainage are simultaneous indications Migration of small lymphoid cells into the blood stream may perhaps account for part of the thymus weight decrease (11) The following reduction in the number of dying cells shows that a major proportion of cortisol sensitive thymus cells have already disappeared This agrees with the fact that thymus weight remains nearly constant for from 6 to 12 hours From 12 to 24 hours thymus weight is kept constant in spite of a rise in the number of dying cells Considering that the number of mitotic figures is very low in the 24 hour group the increase in cell death rate must be interpreted in the light of recent studies (11 13) that show blood borne cells entering the thymus during the course of steroid or X ray induced thymus involution There is some reason to believe that these blood borne cells arise in the bone marrow since this organ remains rather insensitive to a single injection of cortisol (26) However it is possible that the observed mitotic activity in the 12 hour group may partly explain the constancy of thymus weight during the 12 to 24 hours period

The further increase in the death rate of thymus cells along with a thymus weight decrease from the 1st to the 3rd day seems to show that blood borne cells are rather short lived Cortisol induced inhibition of DNA replication and cell mitosis destroy such cells in the thymus environment Autoradiographically the short life span of the majority of the primitive thymic lymphoid cells (about 8 hours) has been shown in numerous studies (12 17 19) At the last day of the experimental period (Fig 2) the number of dying thymic lymphoid cells reaches control level At this point the thymus weight is normal and histologically the gland shows complete regeneration

SUMMARY

One and 2 months old albino mice male and female were injected intraperitoneally with cortisol The Dye Exclusion Test disclosed a striking decay of lymphoid cells in the thymolymphatic system Decay continued several days after injection The thymus was more sensitive to cortisol than various investigated lymph nodes In the thymus the percentage of stained cells increased up to 4 times the control level when the effect of cortisol was maximal the percentage of stained lymph node cells increased up to 2½ times the control level

Findings indicate that no simple relationship exists between the number of pyknotic cells and the stained cells during cortisol induced thymus involution using traditional criteria and the Dye Exclusion Test respectively Only during the first 12 hours after injection the number of pyknotic cells exceeds normal values The number of stained cells reached normal levels at the time when weight and histological appearance of the thymus was normalized

The number of dead and dying lymphoid cells measured with the Dye Exclusion Test appears to be a useful parameter in kinetic studies of lymphoid cells during cortisol induced involution and restitution of the thymus gland

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ABNORMAL BILE DUCT EPITHELIUM IN LIVER BIOPSIES WITH HISTOLOGICAL SIGNS OF VIRAL HEPATITIS

By

HEMMING POULSEN and PER CHRISTOFFERSEN

Received 15 1 69

During recent years abnormal bile duct epithelium in diseases of the liver has attracted considerable attention Some workers (*Popper et al* 1962) are of the impression that it is an important readily diagnostic finding in primary biliary cirrhosis while others (*Bianchi* 1967 *Wepler & Wildhirt* 1968) though only in a smaller number of cases have described it in chronic hepatitis

The main object of the work presented here has been to obtain an impression of how frequently bile ducts with abnormal epithelium are encountered in a consecutive series of liver biopsies all showing typical changes of the parenchyma as in viral hepatitis

MATERIAL

The material consists of a total of 83 percutaneous liver biopsies all with a histological appearance as seen in viral hepatitis characterized by 1) focal liver cell necroses 2) condensation of reticulum fibers 3) focal Kupffer cell proliferation 4) focal infiltration with lymphocytes 5) ballooning of liver cells 6) pleomorphism of nuclei 7) mitoses and/or liver cells with two or three nuclei and 8) acidophilic bodies These changes are mainly centrilobular in distribution and are often associated with central vein oedema

The biopsies have been selected from a total material of 1 582 consecutive biopsies received at the pathological department Kommunehospitalet during the period 1 10 1962–1 10 1968

The biopsies were obtained by the method of *Menghini* and have been received from six medical departments (*Copenhagen Liver Study Group*) The biopsies are 1 1 4 mm thick and 1 5–4 5 cm long

The tissue has been fixed in neutral formalin and imbedded in paraffin. In the beginning of the period 10–1 sections were cut on a sledge microtome while later on 40 serial sections were cut on a rotary microtome The sections are approximately 5 µm in thickness

The assesment has been performed by the two authors in close cooperation without any previous knowledge of the clinical data on haematoxylin and eosin and v Gieson Hansen stained sections as well as on sections stained for reticulin

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Our thanks are due to *K Winkler* M D Head of dept for Clinical Physiology Kommunehospitalet for evaluation of clinical data

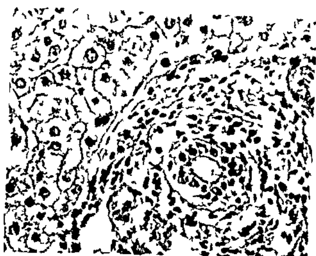


Fig 1

Portal area containing a bile duct with slightly abnormal epithelium The cells are somewhat enlarged with lightly stained partly vacuolated cytoplasm and here and there show karyopyknosis There is only a slight inflammatory infiltrate in the connective tissue consisting of lymphocytes and histiocytes × 140

In addition to bile ducts with abnormal epithelium, the degree of hepatitis (1-3 according to the number of focal liver cell necroses and the variation in size of the hepatocytes and their nuclei) and the degree of fibrosis cholestasis bile duct proliferation portal inflammation and piece meal necroses (o 3) have been recorded Cholestasis has been registered when indisputable intercellular or intracellular bile thrombi have been demonstrated

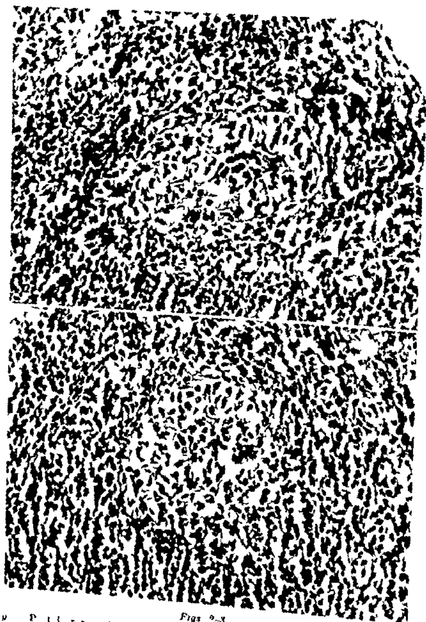
RESULTS

Abnormal bile duct epithelium has been demonstrated in 14 out of the 83 biopsies These 14 biopsies are in the following pages designated group 1 while the remaining 69 are called group 2

The epithelium of the bile ducts in the portal areas is when it is abnormal enlarged swollen with lightly coloured cytoplasm partly confluent with ill defined cellular limits and generally with areas with an indistinct basement membrane and infiltrated with lymphocytes and sometimes plasma cells and granulocytes The lumen is completely obliterated in four cases partly in three In the vicinity there is a considerable infiltration with lymphocytes and histiocytes but no necrosis or occurrence of bile pigment Only in a few cases signs of development of germinal centres are observed In eleven cases there is proliferation of bile ducts in the same portal area as in which abnormal bile ducts are seen

In serial sections it is found that the changes are segmental and often only embrace part of the circumference of the bile duct In each biopsy only one or possibly two or three abnormal bile ducts are seen

In one biopsy only quite slight changes are seen (Fig 1) in the others the changes have been quite pronounced (Fig 2 and 3)



Figs [illegible]

[illegible] ntaining a [illegible] duct with markedly abnormal epithelium The [illegible] enlarg[illegible] lated and [illegible]how karyopyknosis and karyorrhexis and are in add[illegible] infiltrated by lymphocytes The lumen contains cellular [illegible] and [illegible] nounced infiltration with inflammatory cells [illegible] 350

[illegible] with partly [illegible] lumen × 350

TABLE 1

The Table Shows the Distribution According to Sex, Age and Different Histological Qualities in the 14 Biopsies with Abnormal Bile Duct Epithelium (Group 1)

				Parenchyma			*Portal tracts*				
Patient no	Liver biopsy no	Sex	Age	Degree of hepatitis	Degree of cholestasis	Degree of piece meal necrosis	Degree of fibrosis	Degree of inflammation	Degree of typical germinal centres	Degree of bile duct proliferation	Degree of periductal fibrosis
1	1421	♀	65	++	0	0	0	++	0	0	0
2	1430	♀	56	++	0	+	++	++	0	++	0
3	1476	♀	50	+++	0	0	+	++	0	0	+
4	1555	♀	67	+	0	+	+	++	0	+	0
5	1563	♀	48	++	0	++	+	+++	0	++	0
6	1651	♀	77	+++	0	0	+	+	0	0	0
7	1858	♀	80	++	+	+	++	++	+	+	+
8	1934	♀	4[illegible]	++	0	+	++	+++	0	+	+
9	2176	♀	59	+	+	0	0	+	0	0	+
10	2304	♀	45	++	+	+	0	++	0	++	0
11	2429	♀	82	++	0	+	+	++	0	+	0
12	2717	♀	6	++	0	++	++	++	0	++	0
13	2961	♀	45	++	0	0	++	++	0	++	++
14	3101	♀	78	++	0	+	+	++	0	+	0

As far as may be judged on the basis of unaffected parts of the bile ducts the lesion has in all cases affected ducts of medium size This is further supported by the size of the concomitant branch of the portal vein and the central position of the pathological ducts in the portal areas

Table 1 shows the distribution to sex age and different histological qualities in the 14 biopsies with abnormal bile duct epithelium While all 14 biopsies in group 1 come from women the sex distribution in the remaining part of the material (69 biopsies) is 33 men and 36 women

The age distribution in group 1 is between 45 and 82 years with an average of 62 years while group 2 has an age distribution between 16 and 81 years with an average of 52 years

Only two biopsies in *group 1* show slight hepatitis while the other twelve present moderate or severe hepatitis Submassive hepatic necrosis has not been seen In three of the biopsies there is a slight cholestasis In none of these three cases is the lumen of the bile ducts with abnormal epithelium seen to be obliterated

More than half (in all nine) show piece meal necroses of which two are pronounced There is inflammation of the portal tracts in all biopsies two only in light degree and portal fibrosis in twelve In five biopsies the portal fibrosis is heavy with partial disruption of the peripheral portions of the lobules in four cases Nodular regeneration has not been demonstrated Only in one biopsy typical germinal centres are found In ten biopsies there is proliferation of the bile ducts This is pronounced in five cases Periductal fibrosis is demonstrated in six biopsies

The degree of hepatitis and the frequency and degree of piece meal necrosis portal fibrosis and inflammation as well as bile duct proliferation is greater in group 1 than in group 2 A comparison of these qualities is shown in Table 2

In *group 2* a total of 18 biopsies with cholestasis has been found Of these 16 show light and 2 moderate cholestasis None contain germinal centres and only one has periductal fibrosis Cholestasis is in all cases—both in group 1 and 2—centrilobular Bile lakes have not been *demonstrated*

Furthermore 64 cases with portal inflammation have been found in group 2 Five cases were without inflammatory cells in the connective tissue

In ten biopsies submassive necroses defined as larger coherent liver cell necroses are found The submassive necrosis is in all cases *localized* centrilobularly and there is only a slight inflammatory cell *response* to it In eight cases the submassive necrosis is found along with a moderate or severe hepatitis in two cases with a light hepatitis Only one case is without portal *fibrosis* (one of the two cases with light hepatitis)

TABLE 2

A Comparison of the Histological Qualities in Group 1 (14 Biopsies with Abnormal Bile Duct Epithelium) and Group 2 (69 Biopsies without Demonstrable Abnormal Bile Duct Epithelium)

	Degree of hepatitis			Degree of piece meal necrosis			Degree of portal fibrosis			Degree of portal inflammation				Degree of bile duct proliferation		
	+	++	+++	0	+	++	0	+	++	0	+	++	+++	0	+	++
Group 1 (14 biopsies)	2 (14%)	10 (71%)	2 (14%)	5 (36%)	7 (50%)	2 (14%)	2 (14%)	7 (50%)	5 (36%)	0 (0%)	2 (14%)	10 (71%)	2 (14%)	4 (28%)	5 (36%)	5 (36%)
Group 2 (69 biopsies)	36 (52%)	25 (36%)	8 (12%)	54 (78%)	13 (19%)	2 (3%)	25 (36%)	34 (49%)	10 (15%)	5 (7%)	48 (70%)	15 (22%)	1 (1%)	44 (64%)	19 (28%)	6 (9%)

The figures refer to the number of biopsies in each subgroup of the five qualities
In brackets is given the percentage which the number is of the total subgroup

SUMMARY

The concentrations of plasma angiotensinogen and renin were markedly changed after experimentally induced changes in renal morphology and function. A mutual regulation of the angiotensinogen concentration and the renin concentration was only probable in so far as most of the groups of differently pretreated rats showed an inverse correlation between plasma angiotensinogen and plasma renin while this was not a regular finding when the values from the individual rats were compared.

The increases in angiotensinogen concentration with time were so similar after nephrectomy and ureterligation that a common cause of stimulation is probable. Depletion of plasma renin seems not to be the cause as the plasma renin concentration was much lower in nephrectomized than in ureterligated rats. As both nephrectomized and ureterligated rats had the same degree of uraemia the uraemic state could be the cause of the increase in plasma angiotensinogen. The finding of still higher angiotensinogen concentrations in partially corticectomized or partially medullectomized rats indicate, however, that uraemia in these cases can at least only be partly responsible for the increase in plasma angiotensinogen.

Increase in plasma angiotensinogen was further found to be provoked by hypoxia. That the stimulations were due to different causes in the uraemic nephrectomized and in the non-uraemic hypoxic rats was shown by differences in the increase in plasma angiotensinogen with time in nephrectomized (or ureterligated) and hypoxic rats and by a summation of the effects on the angiotensinogen concentration in hypoxic + nephrectomized (or hypoxic + ureterligated) rats.

Plasma renin concentration was extremely low in nephrectomized and partially corticectomised rats, about half the normal in ureterligated hypoxic and most of the partially medullectomised rats, elevated in rats with spontaneous hypertension and normal or markedly elevated in renal hypertensive animals.

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NOTE ADDED IN PROOF

In recently published papers increase of renin substrate was *found in ureterligated* (*K Hirasawa et al* Jap Circ J *32* 1591–1595 1968) and in hypoxic rats (*A B Gould & S A Goodman* Abstracts I IV Int Congr Nephrol Stockholm page 200 1969) The hypothesis that a feedback normally operates tending to maintain plasma renin activity constant as substrate levels alter was further discussed *in studies of S L Skinner et al* (Clin Sci *36* 67–76 1969) *E Rosset & R Veyrat* (Acta endocr Suppl *138* 122 1969) and *J Menard & P Milliez* (C R Acad Sc Paris *268* 1749 1752 and 2710–2713 1969)

Acta path microbiol scand 77 399 404 1969

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A CYTOCHEMICAL METHOD FOR THE STUDY OF BILE CANALICULI IN FINE NEEDLE ASPIRATES OF THE LIVER

By

CURT WASASTJERNA

Received 12 v 69

Although diagnostic fine needle puncture of the liver is an easy and safe procedure the information obtained is rather limited in scope if the cytological specimens are stained by standard methods such as May Grunwald Giemsa or Papanicolaou Tumour cells are detectable in a high percentage of cases with cancer metastases (*Soderstrom* 1966 *Lundquist* 1968 a) and the cytological diagnosis of hepatitis is practicable in many cases (*Lundquist* 1968 b) but the diagnostic value of histological specimens is usually superior for other purposes However histological biopsy with punch needles is not without risk (*Sherlock* 1968) Accordingly different staining procedures should be tried to derive more information from cytological specimens The enzymatic activity of the liver cells is high and valuable results can thus be expected from enzyme cytochemical methods

The bile canaliculi are some of the most interesting structures of the liver Their function is important under normal circumstances and some forms of liver disease result from changes in the canaliculi (*Schaffner & Popper* 1959 *Popper* 1968) For the most part the canaliculi have been studied by electron microscopy (*Popper* 1967) For light microscopy the canaliculi can be visualized by staining frozen sections for alkaline phosphatase (*Gomori* 1941 *Wachstein* 1959 *Bitensky* 1967) or for ATPase (*Wachstein & Meisel* 1957 *Novikoff et al* 1958 *Wachstein* 1959) The latter method provides better results but is rarely applied in routine histology for clinical purposes

Great value would be attached to a simple staining method for the evaluation of bile canaliculi in cytological specimens Among a number of cytochemical procedures tested staining for the demonstration of an amino acid naphthylamidase was found to give the best results

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Aided by a grant from the *Sigrid Jusélius Foundation* A part of the clinical material was very kindly supplied by the staff of the Aurora Hospital and of the Second and Fourth Surgical Departments of the Helsinki University Central Hospital

METHOD

Cytological liver specimens were obtained from hospital patients with the instrument designed by *Franzén* in 1955 (*Franzén et al* 1960) The outer diameter of the needle is 0 7 mm Large livers were often punctured by the abdominal approach. In other cases the site of puncture was usually the 9th intercostal space in the mid axillary line A small drop of tissue fluid was drawn into the needle and smeared on glass slides by the conventional haematological technique The smears were allowed to dry at room temperature In addition to special stainings, one slide was always stained by the May Grunwald Giemsa method

Staining for the demonstration of amino acid naphthylamidase was effected by a modification of the procedure described for haematological purposes by *Rutenberg & Rosales* (1966) The smears were fixed *in a cold (about 4 C) mixture of equal* parts of chloroform and acetone for 3 minutes rinsed with running water and dried They were incubated in the following solution for 1 hour at 37 C

Acetate buffer 0 1 M pH 6 5	25 ml
Sodium chloride solution 0 85 per cent	20 ml
Substrate stock solution	2 5 ml
KCN solution 0 02 M	2 5 ml
Fast Garnet GBC	25 mg

The substrate stock solution was prepared by dissolving 80 mg of alanyl 4-methoxy ? naphthylamide (Cyclo Chem Corp) in 10 ml of distilled water After incubation the smears were rinsed in distilled water for 2 *minutes and counter* stained with Haemalum for 5 minutes The smears were then rinsed with tap water dried and covered with glycerol gel and a cover slip

RESULTS

The positive reaction attained by application of this method is a brilliant red colour Normal liver cells were either negative or displayed a slight granular reaction usually concentrated to the periphery of the cell The colour of the cytoplasm was light yellow both after the ordinary staining procedure and in controls incubated without substrate No red colour was seen in the controls The enzyme activity was somewhat diminished by the fixation used If the smears were fixed for shorter periods or without chloroform the intracellular activity was more marked but the background staining was more pronounced and the contrast poorer

The amino acid naphthylamidase of the liver canaliculi seems to be rather stable In most cases the smears were fixed a few hours after the aspiration and stored for some days at + 4 C until stained *however* comparable results were obtained with unfixed smears stored at room temperature for up to 5 days The colours of the stained smears did not change in about 6 weeks but a slight distortion of the pattern was discernible as a result of shrinkage of the glycerol gel used for mounting the cover slips All the other mounting media tested as well as immersion oil on uncovered smears have been found to dissolve the stain almost immediately Most of the enzyme activity was concentrated in the bile canaliculi These were distinctly visualized as about 1 5 μm wide bright red bands forming networks between the cells (Figs 1 2) The canaliculus border was sharp Usually a black crystallized structure was visible in some parts of the preparations mostly

in thicker tissue fragments but the canaliculi were never disturbingly obscured. No black precipitation occurred if Garnet GBC was replaced by fast blue B salt in the incubation solution. The canaliculi were distinctly visualized with the latter coupling agent, but the contrast was not as good as with Garnet GBC and details of the canaliculus wall were not as well distinguishable. Therefore the author prefers Garnet GBC. Some smears were stained for the demonstration of alkaline phosphatase by an azo coupling method (*Merker & Heilmeyer* 1960). The canaliculi were not visualized by this procedure.

Eighty aspirates have been studied. The results in liver disease will be reported in detail when a more extensive material has been compiled and analysed and after the completion of some animal experiments. However, the first impression is a wide variability of canaliculus pattern in different liver disorders. In mild cases of virus hepatitis the walls have seemed to be irregular or broken. In more severe cases the continuity of the canaliculus system was lost, and in the most severe cases only irregular red patches were observable around the periphery of liver cells (Fig. 3). In the few cases of cirrhosis studied to date the granular intracellular enzyme activity has been increased. The appearance of the canaliculi varied between the normal pattern and distorted bands. In some areas no canaliculi were seen, and other cell groups seemed to contain an irregular proliferation of short canaliculi. In extrahepatic obstructive jaundice the canaliculi were dilated and their borders more diffuse than is normally the case (Fig. 4). On occasion the red colour covered parts of the surrounding cells, but as a rule the continuity of the network was preserved.

DISCUSSION

The fine needle aspiration of liver cells is safe and almost painless to the patient. The staining method described here is fast and simple and suitable for routine use. The bile canaliculi are stained as distinct red bands and can thus be studied in great detail. They seem to be very resistant. Sometimes the adjoining cells have been torn off, but the canaliculi are still intact.

The bile canaliculi are specialized parts of the liver cell membranes (*Popper* 1968) and seem to contain a much higher activity of amino acid naphthylamidase than other parts of the cells. No effort has been made to find out whether the enzyme activity is specific for a particular amino acid. This is probably not the case, but the substrate now used is evidently highly suitable for morphological studies. In normal liver the staining of the canaliculi was remarkably uniform and exhibited a distinct pattern on microscopic study at high magnification. It may thus be concluded that the enzyme activity is probably mainly a characteristic of the canaliculus wall and not, at least exclusively, produced by bile in the canaliculi. If fresh human bile obtained from

T drains of cholecystectomized patients was smeared on albumin covered glass slides and these were stained by the present method only a faint pink colour was distinguishable

The negative results of staining for alkaline phosphatase indicate that this enzyme is rinsed away during the fixation and staining procedure It is probably mainly a component of the bile In histological specimens of normal dog livers *Aronsen et al* (1968) found alkaline phosphatase activity in the epithelium of the ductules and in the peri portal canaliculi and sinusoids Liquid tissue components are better preserved in histological specimens than in thin smears but small intra and extracellular details are often better distinguishable in smears

The morphology of the canaliculus network is disturbed in liver disease In most cases so far studied the pathological pattern could be easily distinguished from the normal morphology However the pattern varied considerably from case to case in similar disorders occasionally even from one cell group to another in the same smear Accordingly a morphological diagnosis was not always possible Nevertheless the study of the canaliculus morphology contributed to the final diagnosis in many cases More experience is required but it can already be stated that the method provides valuable information about the canaliculi and it may thus be a useful diagnostic procedure for the examination of patients with liver disorders

SUMMARY

A simple enzyme staining method for visualization of the bile canaliculi in cytological liver specimens is described Alanyl 4 methoxy 2 naphthylamide is used as substrate and Garnet GBC as coupling agent In normal liver the canaliculi are seen as regular red bands against a light yellow background In liver disease the canaliculus network is disturbed and several different patterns are encountered in different diseases The intracellular activity varies between negative and a moderate granular reaction

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THE ULTRASTRUCTURE OF BETA CELL ISLET TUMOUR IN A DOG

By

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Received 8 iv 69

Staining methods used in the identification of normal alpha and beta islet cells do not always produce clearly defined tinctorial reactions when applied to islet cell tumours of man (*Frantz* 1959 *Greider & Elliot* 1964 *Georgsson & Wessel* 1967 *Toker* 1967a) or dog (*Wilkinson* 1964 *Marcus et al* 1964) At ultrastructural level however the neoplastic alpha and beta cells of man can be differentiated without histochemical techniques (*Lacy* 1959 *Lazarus & Volk* 1962 *Bencosme et al* 1963 *Greider & Elliott*, *Toker* 1967 a b)

Islet cell tumours of animals verified with light microscopy are infrequently encountered in the available literature The cases reported previous to the papers of *Bullock* (1965) and *Rouse & Wilson* (1966) are included in the review of literature by *Roulatt* (1967) Altogether twenty four insulomas of dog are on record so far This electron microscopic study reveals that beta cell islet tumour of dog can be identified by the ultrastructure of cytoplasmic granules in the tumour cells

CASE HISTORY AND METHODS

A ten year old Finnish Harrier bitch was subject to attacks of exhaustion under exercise Clinical examination revealed a mammary tumour and lesions in the heart and pancreas were suspected Without more detailed examinations the dog was euthanatized and an immediate autopsy carried out A whitish fairly firm spherical tumour 6–8 mm in diameter was noted superficially in the middle of the duodenal portion of the pancreas The macroscopic and microscopic investigations revealed a non metastasizing adenocarcinoma in the mammary gland in addition to the pancreatic tumour

For light microscopy formalin fixed tissue from the pancreatic tumour non neoplastic pancreas and from the *pancreas of a healthy* dog were embedded in paraffin cut at 5 μ and stained with Gomori's (1939) aldehyde fuchsin and chrome haematoxylin phloxine by *Hansen*'s (1949) method for beta granules with *Laynay*'s method for zymogen granules and with haematoxylin eosin (H & E) Van Gieson and periodic acid Schiff (PAS)

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This investigation was supported by a grant from *L ak tehdas Orion Scientific Research Foundation*

For electron microscopy small blocks of tissue of the pancreatic tumour and of the non neoplastic pancreas were fixed at 4 C in 2 5 per cent glutaraldehyde in 0 1 M phosphate buffer for 2 hours washed in the same buffer plus 10 per cent sucrose and postfixed at 4 C in 1 per cent osmium tetroxide in the same buffer for 2 hours. All tissues were rapidly dehydrated in a graded series of ethanol solutions and embedded in Epon (*Kay* 1965) Sections cut at 1 μ were stained with 1 per cent basic fuchsin in 50 per cent acetone for light microscopic identification of tissue in blocks (*Winkelstein et al* 1963) Thin sections were cut with glass knives using an *Ultrotome* ultramicrotome mounted on copper grids stained with aqueous solutions of uranyl acetate (*Kay*) and examined with an *Akashi* electron microscope

RESULTS

Light Microscopy

The encapsulated tumour presented an abundant stroma consisting of anastomotic bands of fibrous tissue and thin walled blood capillaries The parenchyma was composed of irregular islets and cords of cells In an area of the tumour cell groups resembling acinar and tubular structures were noted (Fig 1) A homogenic PAS positive substance filled the lumen of some tubular looking structures Immediately around this tissue appearing exocrine the neoplastic islet cells were pleomorphic presenting an irregular nucleus and a scanty often finely vacuolated cytoplasm Mitotic figures in these cells were fairly frequent The exocrine looking tissues inside the tumour seemed to originate from neoplastic islet cells instead of being entrapped by the expanding tumour

The greatest part of the tumour consisted cords of predominantly rectangular cells with a small spherical or elongated nucleus The cytoplasm was abundant faintly eosinophilic and finely granular (Fig 2) With specific stains only a few tumour cells presented a profusion of beta granules like normal beta cells in islets surrounding the tumour Moreover occasional beta granules could be demonstrated in some tumour cells but most cells were devoid of them

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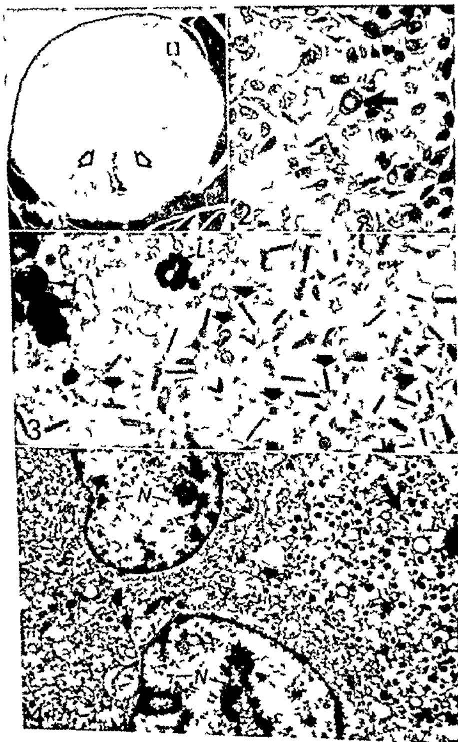
Figs 1 4

Fig 1 Low power photomicrograph showing the capsule with adjacent pancreatic parenchyma and general topography of beta cell islet tumour The arrows point to an area with dark stained structures resembling exocrine acini The square area in the adjoining section of the tumour is shown in detail in Fig 2 H & E × 13

Fig 2 High power photomicrograph showing a cluster of well differentiated rectangular neoplastic islet cells Inclusion like structure with granular inner matrix encircled by membrane in the nucleus of a tumour cell (arrow) Van Gieson × 540

Fig 3 Cytoplasm of beta cell from *non neoplastic* islet presenting numerous beta granules (*e g* arrows) Intrasaccular rectangular bar shaped or spherical cores of granules surrounded by clear space L lipid body × 14 000

Fig 4 Sections of three adjacent tumour cells In the cell to the right a cluster of beta granules with predominantly spherical core separated by clear space from surrounding smooth membrane Bar shaped beta granule (long arrow) In cells to the left a few spherical beta granules (short arrows) N nucleus G Golgi zone L lipid body × 11 000


1
2
3
N
N

A structure resembling an inclusion and consisting of a finely granular eosinophilic matrix occurred in many nuclei (Fig. 2). The roundish structure enclosed in a membrane was located in the nucleoplasm sometimes in contact with the nuclear membrane. Similar structures occurred in the nuclei of some non neoplastic islet cells. No other significant changes were noted in the pancreatic parenchyma.

Electron Microscopy

The different cells in the non neoplastic pancreatic islets were identified according to the descriptions of ultrastructure in the relevant literature (*Lacy* 1957, 1961; *Herman et al.* 1964; *Munger et al.* 1965; *Sato et al.* 1966). The beta cells presented numerous cytoplasmic granules consisting of an electron opaque central core separated by a translucent halo from the limiting agranular membrane of a spherical profile. The profile of the central core was rectangular, bar shaped or sometimes roundish (Fig. 3).

A varying number of cytoplasmic granules of the same size as the normal beta granules occurred in the tumour cells (Fig. 4). The roundish or bar shaped cores were surrounded by a broad translucent space and limited by a smooth membrane of a spherical profile (Fig. 5). Most granules were similar to the beta granules in the non neoplastic beta cells. The relative number of spherical beta granules however was greater in the tumour cells than in the non neoplastic beta cells (Fig. 6).

Most cells presented only few granules, an abundance of membraneous structures and unattached ribosomes. Rough endoplasmic reticulum formed parallel profiles and dilated cisternae and occasionally separate spherical structures (Figs. 5 and 7). Cisternae and membraneous structures of smooth endoplasmic reticulum were distributed at random in the cytoplasm. Aggregates of cisternae with a smooth surface sometimes resembled the Golgi complex (Fig. 4). The size and structure of mitochondria was not uniform (Fig. 8). A few lipid bodies occurred in the cytoplasm (Fig. 4).

Chromatin had a tendency to marginalization (Fig. 9). Nuclei in

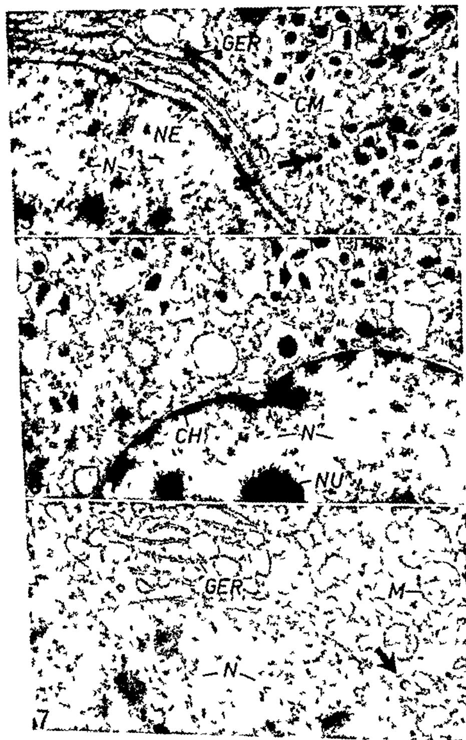
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Figs. 5–7

Fig. 5. Tumour cell poor in beta granules (left). Spherical beta granule (long arrow). Part of parallel array of granular endoplasmic reticulum (ER) in area adjoining nucleus (N). NE, nuclear envelope; CM, cell membrane. Numerous beta granules in the cell to the right. Bar shaped beta granules (short arrows). × 18 000

Fig. 6. Tumour cell with many spherical and a few bar shaped (arrows) beta granules. N, nucleus; Nl, nucleolus; Ch, chromatin. × 21 000

Fig. 7. Tumour cell poor in beta granules. Spherical beta granule (arrow). An abundant rough endoplasmic reticulum (ER) forming parallel arrays, cisternae and spherical structures. N, nucleus; M, mitochondria. × 21 000


GER
CM
NE
N
CH
N
NU
GER
M
N
7

A structure resembling an inclusion and consisting of a finely granular eosinophilic matrix occurred in many nuclei (Fig 2) The roundish structure enclosed in a membrane was located in the nucleoplasm sometimes in contact with the nuclear membrane Similar structures occurred in the nuclei of some non neoplastic islet cells No other significant changes were noted in the pancreatic parenchyma

Electron Microscopy

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Most cells presented only few granules an abundance of membraneous structures and unattached ribosomes Rough endoplasmic reticulum formed parallel profiles and dilated cisternae and occasionally separate spherical structures (Figs 5 and 7) Cisternae and membraneous structures of smooth endoplasmic reticulum were distributed at random in the cytoplasm Aggregates of cisternae with a smooth surface sometimes resembled the Golgi complex (Fig 4) The size and structure of mitochondria was not uniform (Fig 8) A few lipid bodies occurred in the cytoplasm (Fig 4)

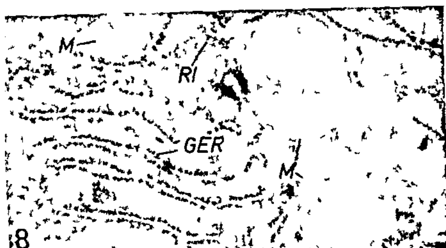
Chromatin had a tendency to marginalization (Fig 9) Nuclei in

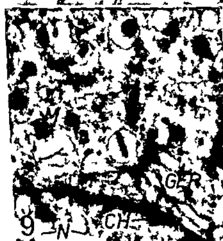
Figs 5 7

Fig 5 Tumour cell poor in beta granules (left) Spherical beta granule (long arrow) Part of parallel arrays of granular endoplasmic reticulum (ER) in area adjoining nucleus (N) NE nuclear envelope CM cell membrane Numerous beta granules in the cell to the right Bar shaped beta granules (short arrows) × 18 000

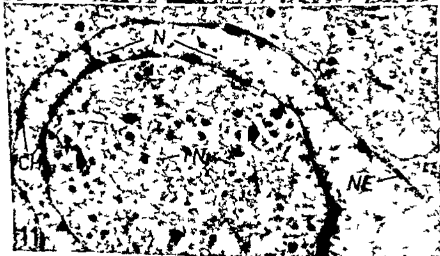
Fig 6 Tumour cell with many spherical and a few bar shaped (arrows) beta granules N nucleus NU nucleolus CH chromatin × 21 000

Fig 7 Tumour cell poor in beta granules Spherical beta granule (arrow) An abundant rough endoplasmic reticulum (ER) forming parallel arrays, cisternae and spherical structure N nucleus M mitochondria × 21 000


M
RI
GER
M
8


M
GER
9
N
CH


N
10


N
CH
N
NE
11

the invading fraction of cytoplasm is only apparently separate and does not form a true intranuclear structure

SUMMARY

A light and electron microscopic investigation of a beta cell islet tumour in a ten year old Finnish Harrier is reported

The encapsulated tumour had a general morphology characteristic of an endocrine neoplasm of relatively low malignancy With specific stains beta granules could be demonstrated in a few tumour cells

Tumour cells presented a regular ultrastructure and an abundance of cytoplasmic organelles Cytoplasmic granules of the same ultrastructure as normal beta granules occurred in small numbers Spherical beta granules dominated in the tumour cells whilst rectangular beta granules were frequent in the examined non neoplastic beta cells

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TABLE 2

Results of Oral Inoculation into Mice of Serial Dilutions of a Suspension Containing 50 000 Cysts/ml Dosage 0.2 ml/Mouse

Inoc dilut	No of mice inoc	No of mice dead/inoc	No of mice pos/inoc		No of pass mice pos/inoc	
			Dye test	Brain cysts	Dye test	Brain cysts
Undil	6	6/6	–	–	24/24	24/24
1:10	6	1/6	5/6	5/6	4/4	4/4
1:10^{2}	6	0/6	6/6	6/6	–	–
1:10^{3}	6	0/6	4/6	4/6	–	–
1:10^{4}	6	0/6	0/6	0/6	–	–
1:10^{5}	6	0/6	0/6	0/6	–	–
1:10^{6}	6	0/6	0/6	0/6	–	–
1:10	6	0/6	0/6	0/6	–	–

inoculation. Blood was obtained from four; two had a titre of 1:10 (9th day) and two had 1:50 (11th day) in the dye test. Six mice received 1 000 cysts and one of these died 10 days after inoculation. All surviving mice were bled and examined in the dye test 18 days after inoculation. The remaining five of six mice that received 1 000 cysts each were positive with titres of 1:250, and so were six mice that received 100 cysts. Six mice were given 10 cysts each and four became positive; two had a titre of 1:250 and two had 1:50. The mice in the remaining groups were all negative including six mice that received a dosage equivalent to one cyst per mouse. The passage mice were positive, most of them with a titre of 1:1250, a few with 1:250, in the dye test. Numerous *Toxoplasma* tissue cysts were seen in the brains of all serologically positive mice, and none were observed in the negative mice. All control mice were negative.

This experiment was repeated, but this time the mice were ino-

TABLE 3

Results of Intraperitoneal Inoculation of Serial Dilutions of a Suspension Containing 50 000 Cysts/ml Dosage 0.2 ml/Mouse

Inoculum dilution	No of mice inoculated	No of mice dead in c	No of mice pos./inoc	
			Dye test	Brain cysts
Undiluted	6	0/6	6/6	6/6
1:10	6	0/6	6/6	6/6
1:10	6	2/6	4/6	4/6
1:10^{3}	6	0/6	5/6	5/6
1:10^{4}	6	0/6	1/6	1/6
1:10	6	0/6	0/6	0/6

Two mice died 18 and 19 days after inoculation respectively but were not examined

culated intraperitoneally Results are presented in Table 3 The mice were bled and examined 22 days after inoculation Six mice that received 10 000 cysts were positive with titres of 1 250 in the dye test and so were six mice that received 1 000 cysts each Of the six mice that were inoculated with 100 cysts each two died 18 and 19 days after inoculation respectively These two mice were not examined but the remaining four were positive Six mice received 10 cysts each and five became positive In the group that was inoculated with a calculated dosage of one cyst per mouse one out of six became positive with a titre of 1 250 in the dye test All the mice in the last group remained negative and so did all control mice *Toxoplasma* tissue cysts were demonstrated in the brains of all serologically positive mice whereas none were seen in the dye test negative mice

Single cysts were micro isolated and inoculated intraperitoneally into each of four mice Another four mice were used as controls These mice had been pre bled and examined in the dye test, and were negative with a titre of less than 1 5 They were bled and examined again 18 days after inoculation and all four were positive with titres of 1 250 Numerous *Toxoplasma* tissue cysts were found in their brains The control mice had remained negative

When the four cats in this group were again fed infected mice about three months later and faecal separations were prepared as before it did not result in any cyst formation On microscopical examination of the faecal material no cystic structures were seen nor were any nematode ova observed All the mice inoculated with faecal material remained serologically and parasitologically negative

Group II

Microscopical examination Cysts as described above were found in the faeces of all three cats in this group From cat 6 they were seen in the faeces collected during the second week after feeding while from cats 7 and 8 they were found in the material from both the first and second week Besides the cysts numerous bacteria and fungi were seen But no other protozoan cysts were observed nor were any nematode ova seen

Mouse inoculation The results obtained in this experiment are very similar to the results described in group I (Table 4) Mice inoculated with material containing cysts were dying within 5–13 days The longest time of survival was noted for mice fed material collected during the first week from cat 8 Four out of six mice in that group died after 11–13 days while the remaining two recovered and survived to autopsy after six weeks One mouse out of six fed material from the first week of cat 6 became serologically and parasitologically positive although no cysts were noted in the material on microscopy

A total of 23 passage mice from cat 6 63 from cat 7 and 40 from

TABLE 4

Results of Feeding Mice Faecal Material from 3 Experimentally Toxoplasma Infected Nematode Negative Cats Dosage 1 ml Per Mouse

Cat no	Weekly periods	Cysts*	Mortality of mice		Surviving mice		Passage mice
			Dead/Inoc	Surviv time	DT pos/Inoc	Brain cysts	Pos/Inoc
6	Control	–	0/6		0/6	0/6	
	1	–	0/6		1/6	1/6	
	2	+	6/6	6–10 days		–	23†/24
	3	–	0/6		0/6	0/6	
7	Control	–	0/6		0/6	0/6	
	1	+	6/6	6–7 days	–	–	19/24§ 20/20
	2	+	6/6	5–9 days	–	–	24/24
	3	–	0/6		0/6	0/6	
8	Control	–	0/6		0/6	0/6	
	1	+	4/6	11–13 days	2/6	2/6	16/16
	2	+	6/6	6 9 days	–	–	24/24
	3	–	0/6		0/6	0/6	

* In faecal material

† One mouse died within 24 hours after inoculation and was not examined

§ Five mice died within 12–15 days after inoculation The brain of each was inoculated into four mice

cat 8 were examined in the dye test They were all positive with titres from 1 1250–1 6250 On microscopy of their brains numerous *Toxoplasma* tissue cysts were seen An equal number of control mice were negative

Serological examination of the cats was done at the beginning and at the end of the experiment All three cats had become positive with a rise in dye test titre from less than 1 5 to 1 250

DISCUSSION

Group I

The idea of a possible connection between the cystic organisms described above and *Toxoplasma* infection arose on the basis of the microscopical findings compared with the results of mouse inoculation The cysts were present in fairly large numbers in the material obtained during the first and second period and the mice inoculated with this material died in six or seven days Eight out of these 12 mice had developed *Toxoplasma* antibodies although in low titres before dying The third group however inoculated with cyst free material remained negative This seemed to point to the cyst as causative agent Apparantly there was a correlation between the presence of cysts in the inoculum the clinical illness or death of the mice and the development of *Toxoplasma* antibodies

In order to give further support to the idea of such a correlation the additional experiments were performed Inoculation into mice of serial dilutions of a counted suspension was done twice Oral and intraperitoneal inoculation were used *Sum & Hutchison* (1966) had demonstrated that intraperitoneal inoculation of faecal material from cats could result in *Toxoplasma* infection in mice It is noteworthy that in neither of these experiments did any mouse become positive when inoculated with a dilution representing less than one cyst per mouse On the other hand only one mouse out of twelve became positive when inoculated with a calculated dosage of one cyst However when dealing with so few organisms there are several incidental factors which will influence the outcome Some of the cysts may not be viable A single cyst may adhere to either syringe or needle and not get into the mouse It may also in the case of oral inoculation pass through the intestinal canal without excysting and without causing any infection The one mouse in this experiment which became positive after receiving one cyst was inoculated intraperitoneally When comparing the two routes of infection it is also remarkable that 10 000 cysts are sufficient to kill a mouse when administered orally but not intraperitoneally We cannot give any definite explanation of this but it seems likely that it is due to differences in the process of excystation In the intestinal canal under the influence of the various digestive juices all the organisms may be released within

a short interval of time In the peritoneal cavity the release may be more gradual enabling the mouse to survive the acute stage of the infection

The technique of micro isolation is more suitable for handling single organisms than is the above method The amount of suspending medium is kept at a minimum and the micropipette used for isolation is also used for the subsequent inoculation into the mouse To prevent any possible loss through the intestinal canal the four single cysts were inoculated intraperitoneally and atypical cysts or cysts that seemed to be damaged or non viable were avoided

Removal of all cysts from a suspension of faecal material and inoculation into mice of material cleared of cysts in order to demonstrate the removal of toxoplasmic infectivity with the cysts has been considered repeatedly However for technical reasons we have decided not to attempt such an experiment In some of our suspensions we have seen that bursting of cysts may occur outside of a host Since we do not know how many parasites this would release and what size they would be we feel that neither micro isolation nor filtration would ensure a complete removal of all cystic elements

All results seem to indicate that the cystic organism is the infective agent in these experiments Two alternative possibilities to this are 1) the cyst demonstrated in faeces is not responsible for the infection in mice 2) the infection observed in the mice is not caused by *Toxoplasma gondii*

Arguments against the first proposition are that firstly only bacteria and fungi were observed in the inoculum and no protozoan cysts other than those described were seen Secondly no other living organisms were apparent in the same concentration as the cyst if any other organisms were responsible for the infection this would have to be the case Thirdly *Hutchison et al* (1969) have carried out similar infection experiments in SPF cats in which identical cysts were demonstrated in the faeces

Arguments against the second proposition are that it is highly unlikely that any foreign organism would be capable of forming antibodies and tissue cysts in the brains of mice indistinguishable from those demonstrable in *Toxoplasma* infection

Group II

This experiment was performed in order to reproduce the results in nematode negative and *Toxoplasma* negative cats Unfortunately the cats were not examined post mortem for the presence of nematodes However the faecal material was examined on many occasions by different investigators and nematode ova were never seen Moreover all the cats were given Piperazine Adipate prior to the experiment because this was found to be more effective than Vermiplex So

it seems that in this experiment cyst formation and transmission of *Toxoplasma* were independent of nematodes

Furthermore it was demonstrated that feeding whole mouse carcasses is not necessary to initiate cyst formation Cat 6 was fed infected mouse brains only This means that the influence of any possible cystic forms present in the mouse intestinal epithelium can be excluded

The formation site of the cystic form is unknown However the fact that the cysts in these experiments were produced and shed within 4 5 days seems to indicate that formation might take place in the alimentary tract Studies to investigate this problem are being performed but have not yet been completed

The serological status of the cat prior to the experiment and the way this influences cyst formation is rather interesting and —from an epidemiological point of view—important The presence of dye test antibodies in the cat indicating previous exposure to the parasite does not necessarily interfere with cyst formation On the other hand it can also from the above experiments be concluded that every *Toxoplasma* infected meal will not be followed by cyst formation It looks as if a certain interval of time between exposures was necessary

We are convinced that we are dealing with a new form of *Toxoplasma gondii*, but further studies will be required to fit it into the life cycle of the parasite We are aware of the fact that this cyst is remarkably similar to the isosporan oocyst However we are not prepared to discuss its relationships until more detailed information is available

SUMMARY

A cystic organism exists in the faeces of cats experimentally infected with *Toxoplasma* it seems capable of producing *Toxoplasma* antibodies and *Toxoplasma* tissue cysts in mice An association between this cystic organism and infections in mice by injection of serially diluted inocula and by micro isolations has been demonstrated

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THE POSSIBLE ROLE OF CIRCULATING INTERFERON ON AUTOINTERFERENCE IN MICE INFECTED INTRAPERITONEALLY WITH WEST NILE VIRUS

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Autointerference expresses itself as protection or reduced viral multiplication in hosts inoculated with large doses of virus which in smaller doses is pathogenic and multiplies to high levels (19)

Autointerference in vivo has been observed in connection with intracerebral titration in mice with louping ill (8) yellow fever (23) dengue fever (18 21) and Rift Valley fever (14) viruses and with yellow fever virus inoculated subcutaneously in galagos (22) The phenomenon has probably been observed in several other laboratories without being reported

Attempts have been made to explain the phenomenon as either resulting from the formation of an incomplete virus which might have a blocking effect on suceptible cells (14) or as the consequence of an infection caused by a mixture of non modified and modified virus particles (18) The possible role of interferon on autointerference in vivo does not appear to have been studied

As we in this laboratory have a strain of West Nile virus producing autointerference on intraperitoneal inoculation in mice we have undertaken a study designed to determine what role interferon might play in this phenomenon

MATERIALS AND METHODS

Most materials and methods have been described in detail in previous papers (10 11)

Viruses West Nile virus strain Egypt 101 in its 4th mouse brain passage in this laboratory was used for the infectivity experiments This strain was originally obtained from Casals in 1961 Stocks of West Nile virus were prepared by intracerebral inoculation of virus into suckling mice On the development of symptoms of encephalitis usually after 4 days the brains were removed after preceding exsanguination and ground with sand in 10 parts of PBS (pH 7 3) After centrifugation at 6000 rpm for 30 minutes volumes of 1 ml of the supernatant were stored at –70 C

Semliki forest virus (SF strain Smithburn in its 6th mouse brain passage in this laboratory was used as challenge virus in titration of interferon. This strain was originally obtained from Casals in 1961 The virus stock was prepared as described above under WN

Animals Male and female albino mice of a non inbred strain were used In most

of the experiments the animals weighed 23-26 gm but as given in the text some experiments were made with mice of other weights Eight or ten mice were used in each experimental group

Processing of specimens Removal of blood was done as described previously (10) The pooled blood was centrifuged at 3000 rpm for 10 minutes The serum was pipetted off and used for virus titration and for interferon assay Serum dilutions used for interferon assay were dialysed against Sørensen s buffer pH 2 and after 48 hours at 4 C dialysed back to pH 7 4 After centrifugation at 3000 rpm for 30 minutes the supernatant was used for the assay

Interferon assay was performed as previously described (10) by the plaque inhibition method in secondary cultures of mouse embryonic cells Interferon titres expressed as units per 100 μl of serum were recorded as the reciprocal of the highest dilution which reduced by 50 per cent the number of plaques (SF) counted in the controls i e 50 per cent plaque-depressing dose ($PDD_{50}/100\,\mu l$)

A stock reference preparation of interferon at known titre was used in each assay to detect any changes in the sensitivity of the system

Virus titration The virus content of the serum was determined by intracerebral inoculation into mice aged 2-4 days of 10 fold serial dilutions in PBS The virus titre was expressed as the exponent of the logarithmic ($\log_{10}$) dilution per 20 μl of serum which caused death in 50 per cent of the animals as calculated by the method of *Karber* (12)

The virus doses given in the experiments are determined by intracerebral titration in mice aged 2-4 days

Haemagglutination inhibition test (HI) was carried out using the standard techniques described by Clark and Casals (4) Sera were tested against two HA units beginning at a 1 10 dilution

Neutralization test Sera diluted 1 2 1 10 and 1 50 in PBS were mixed with five doses of LD_{50} West Nile virus After standing at 37 C for 60 minutes the mixture was inoculated intracerebrally in 2-4 day old mice

Steroid Hydrocortisone (Hydrocortisoni acetas NFN Løvens kemiske Fabrik Copenhagen) suspended in water was administered subcutaneously near the tail root in doses of 10 mg per mouse 4 hours prior to inoculation with West Nile virus Control mice were inoculated in the same way with equivalent doses of PBS

Characteristics of viral inhibitor The viral inhibitor found in the serum dilution exhibited the characteristics described in a previous paper (10) The properties are in keeping with mouse interferon as described by other investigators (5)

RESULTS

Autointerference at Different Ages

The autointerference phenomenon was found to be closely correlated to the age and weight of the mice Results obtained in mice of varying

TABLE 1

Mortality in Mice of Varying Age and Weight after Intraperitoneal Inoculation of West Nile Virus in Doses of 10^6 LD_{50} and 10^3 LD_{50}

Weight and age	10^6 LD_{50} No dead/no tested	Mortality %	10^3 LD_{50} No dead/no tested	Mortality %
2 gm/ 2 4 days	10/10	100 %	10/10	100 %
17-19 gm c 3 weeks	13/16	81 %	16/16	100 %
23-26 gm/c 5 weeks	18/40	45 %	35/40	98 %
30-32 gm/c 7 weeks	2/16	13 %	7/16	44 %

age and weight are given in Table 1. As is shown the phenomenon was not seen in mice weighing 2 gm (2–4 days old). In 17–19 gm mice (3 weeks of age) there was 100 per cent mortality with the lowest virus dose and approximately a 20 per cent reduction in mortality when the highest virus dose was given. In older mice presenting a higher resistance against all virus doses an even higher degree of autointerference was found.

Virus and Interferon in Serum during the First 24 Hours Post inoculation

A study was then performed to determine whether there was any difference in viral and interferon content in the serum during the first 24 hours after intraperitoneal inoculation in groups of mice receiving 10^6 and 10^3 LD_{50} doses respectively of West Nile virus. In Table 2 the results of a typical experiment are given.

As shown in the table, interferon could not be demonstrated in the serum at any point of time within the first 24 hours in the group of mice receiving 10^3 LD_{50} and virus could first be demonstrated in the serum after 24 hours.

In the group where 10^6 doses of West Nile virus was given, interferon could be demonstrated in the serum after six hours. Thereafter increasing amounts of interferon were found during the next 12 hours, a maximum being reached after 18 hours. After 18 hours the amount of interferon decreased. Virus could be demonstrated two hours after virus injection but not after six hours. After 12 hours virus could again be demonstrated and thereafter in increasing amounts.

TABLE 2

Virus and Interferon Content of Pooled Serum from 8 Mice during the First 24 Hours after Intraperitoneal Inoculation of West Nile Virus in Doses of 10^6 LD and 10^3 LD_5

Hours after virusinfect	10^6 LD_{50} Virus	10^6 LD_{50} Interferon	10^3 LD_{50} Virus	10^3 LD_{50} Interferon
2	1.5	<4§	<0	<4
6	<0	10		
12	0.35	32	<0	<4
18	2.1	128		
24	1.35	32	1.15	<4

Log LD_{50}/20 µl
§ PDD_{50}/100 µl

Virus and Interferon in Serum During the First Week Post inoculation

In other experiments viral and interferon content in the serum was investigated during the following days in the two groups. Fig. 1 shows

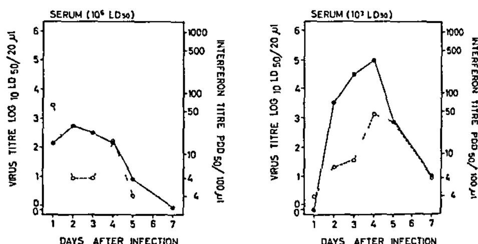


Fig 1

Virus (●——●) and Interferon (○--○) content of pooled serum from 10 mice after intraperitoneal inoculation of West Nile virus in doses of 10^6 LD$_{50}$ and 10^3 LD$_{50}$

the results obtained in one of these experiments In the group receiving 10^3 LD$_{50}$ doses of virus the content of interferon and virus was in accordance with previously performed studies (10) *i e*, increasing through the fourth day and thereafter decreasing In the experiments presented here the viraemia maximum was particulary high Usually maximum occurred during the fourth day with a titre of about 10^4 LD$_{50}$ In the group receiving 10^6 LD$_{50}$ large amounts of interferon could be demonstrated 24 hours after virus injection which is in agreement with the above but during the following days slight or no virus inhibiting activity could be found in the serum Viral content remained at approximately the same level through the fourth day and was consistently lower than in the groups receiving a smaller virus dose In all studies performed a less pronounced viraemia was found from the second day in the groups of mice receiving the larger virus dose

Virus and Interferon in Serum on Autointerference at Different Ages

The relationship between the age of the mice and the appearance of autointerference prompted a comparison of interferon induction and viral content in the serum in mice of various ages in which autointerference is seen Table 3 shows the findings from such a study where intraperitoneal injections of 10^4 LD$_{50}$ doses of West Nile virus were given to groups of mice weighing approximately 17 19 gm and 25 27 gm As will be seen a higher viral and interferon content was constantly found in the younger mice where autointerference was less pronounced

TABLE 3

Virus and Interferon Content of Pooled Serum from Groups of 8 Mice of Different Ages after Intraperitoneal Inoculation of West Nile Virus in Doses of 10^6 LD_{50}

Hours after virus inoculation	Mice weighing 17–19 gm		Mice weighing 25–27 gm	
	Virus	Interferon	Virus	Interferon
24	1 7	32	0 9	16
48	2 5	16	1 5	4
72	2 3	<4	<1	<4
96	1 5	<4	0 7	<4

Absence of Autointerference in Steroid Treated Mice

Previous studies (11) have demonstrated inhibition of interferon induction in mice given steroids prior to virus inoculation Autointerference was therefore studied in steroid treated mice An inoculum corresponding to 10^4 LD_{50} doses of virus was given intraperitoneally in untreated mice and mice treated with 10 mg hydrocortisate 4 hours prior to virus inoculation The untreated mice showed autointerference whereas mortality was 100 per cent in the steroid treated mice Viral and interferon content in serum from two similar groups of mice was studied during the course of infection and is shown in Table 4 As expected a reduction in interferon induction was found in the steroid treated mice Viral content in the serum was found to be almost the same in the two groups during the first 24 hours but hereafter a markedly higher titre was seen in the steroid treated mice

TABLE 4

Virus and Interferon Content of Pooled Serum from 8 Mice Each Mouse being Treated with 10 mg of Hydrocortisate or a Corresponding Volume of Saline 4 Hours before Intraperitoneal Inoculation of West Nile Virus in Doses of 10^4 LD_5

Hours after virus inoculation	Saline		Hydrocortisate	
	Virus	Interferon	Virus	Interferon
2	2 1	<4	1 9	<4
6	<1	12	<1	<4
18	2 3	64	2 1	16
48	1 3	<4	3 0	≦4
72	<1	<4	3 3	<4
120	1 1	4	3 1	<4
144	<1	<4	2 7	<4

Effect of Various Virus Doses in Immune Response

Antibody production in the two groups of mice was elucidated by studying haemagglutinating and neutralizing antibodies responses in individual mice in the two groups Results as regards HI antibodies

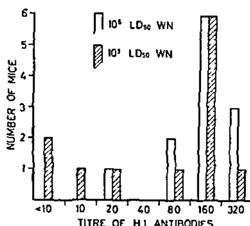


Fig 2

Number of mice with different titres of HI antibody 8 days after inoculation of West Nile virus in doses of 10^6 LD_{50} and 10^3 LD_{50}

eight days after virus inoculation are given in Fig 2 There was no significant difference ($p > 0.10$ rank sum test) in the production of HI antibodies in the two groups Neutralizing antibodies could not be demonstrated eight days after virus inoculation

DISCUSSION

A detailed explanation as to why autointerference appeared on intraperitoneal inoculation with the strain of West Nile virus used here cannot be given on the basis of the present study Among the factors considered to be of importance for the host resistance to viral infection (2) only antibody and interferon production were examined

As regards the role of antibody no significant difference was found in the development of haemagglutination inhibiting antibodies in the two groups of mice receiving different doses of virus Neutralizing antibodies were not found in the serum eight days after viral injection at a time when virus in West Nile infections in mice normally has invaded the central nervous system (10) Antibody production therefore could not have had any decisive influence in reducing mortality in the mice given the larger virus inoculum This is in accordance with the minor role usually attributed to antibody production in the defence mechanism against primary viral infection (2)

In the group where autointerference was observed the interferon content in the serum was found to be relatively high at a time when actual viraemia had not yet developed (the virus demonstrated in the serum two hours after viral injection probably resulted from spillover from the inoculated virus) As protection against neurotropic viral infections in mice often has been demonstrated when interferon has been injected or induced shortly before or after virus injection (3 6

9 16 20) it appears that this early circulating interferon could have played a decisive role in the reduced mortality found in these mice

Suggestive evidence of direct protection against a virus which had stimulated interferon induction as found in the present experiments does not appear to have been obtained previously in *in vivo* experiments

The lack of autointerference demonstrated after steroid treatment further supports the supposition that early interferon induction is of importance for the development of autointerference as early interferon induction was inhibited in these mice These findings do not however provide an unequivocal answer as steroid treatment is supposed to inhibit cellular immunity (17) also thought to be of importance for the resistance to primary viral infection (1)

It is difficult to reach definite conclusions concerning the increased autointerference seen in older mice The amount of circulating interferon and virus was lower in older mice findings that are in agreement with other studies (13 15) Generally the maximum level of viraemia was lower in those groups of mice where autointerference was seen however the present study provides no answer as to whether the less pronounced viraemia was a course of reduced mortality

SUMMARY

Autointerference was observed in mice on intraperitoneal inoculation of West Nile virus and was most pronounced in older animals which exhibit a natural resistance against the virus In mice evidencing autointerference the highest level of circulating interferon was found 18 hours after virus inoculation This is in contrast to mice receiving a smaller virus inoculum where the highest level of interferon first appeared 4 days after virus inoculation Treatment with steroids prior to virus inoculation was associated with an inhibition of interferon induction and autointerference was not observed In older mice where autointerference phenomenon was most pronounced viraemia and the amount of circulating interferon were found to be lower than in younger mice

The induction of early interferon in the mice exhibiting autointerference is considered of importance in the reduction of mortality

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THE EFFECT OF IMMUNOSUPPRESSIVE THERAPY ON THE MURINE LYMPHOCYTIC CHORIO MENINGITIS VIRUS INFECTION

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It has hitherto been demonstrated on a number of occasions that immunosuppressive therapy of adult mice inoculated with lymphocytic choriomeningitis (LCM) virus reduces the lethality rate in the infected mice (16 23) The following immunosuppressive therapy has been used cortisone treatment (17) amethopterine treatment (1 7) and other antimetabolites (7 21) Moreover X ray irradiation (19 23 24) treatment with anti lymphocytic serum (ALS) (6 15 22) or anti mouse IgG (13) have also been used and neonatal thymectomy has also been applied in this infection model (4 18 25)

All these various experiments have given the same clear cut result that the lethality rate of the infection is reduced Following lethal LCM disease large numbers of lymphoid cells are found especially in the meninges and the choroid plexus These infiltrates have been shown to be absent in such treated animals which survive (3 23 24) With regard to the co tolerogenic effect of these procedures the results have been inconclusive Some authors have reported that surviving mice had quite high virus titres for several weeks after the treatment indicating tolerant states but there is no definite information about these conditions except insofar as ALS would seem to be the best immunosuppressive agent in this system also treatment with ALS resulting in tolerance in the treated animals (22)

In order to investigate the effect of cortisone amethopterine (methotrexate) and X irradiation in the LCM/mouse system with respect to the development of tolerance the following experiments were carried out They showed that these treatments only occasionally resulted in a complete and persisting tolerance to the virus (i e no antibody formation and no virus elimination) but often in transient states of incomplete tolerance

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MATERIALS AND METHODS

LCM virus The LCM virus was the strain which has been used in this laboratory for several years It has been grown by intraperitoneal passages in mice The stock virus consisted of 10 per cent clarified mouse spleen homogenate It was kept at —70 C.

Virus titrations These were performed by intracerebral inoculation into young Swiss mice of 0 03 ml of decimal dilutions of the samples These were either blood or organ homogenates *Four mice per dilution were inoculated The LD_{50} titre* was determined after 14 days according to *Kärber*s method (20) and expressed as the reciprocal

The blood samples were taken from the heart in the experiments where the mice were killed Blood samples from surviving animals were taken from the inner canthus of the eye

Complement fixation (CF) This test was performed as described previously (?8) The antigen was made from spleens and livers of tolerant, LCM virus-carrier mice and prepared by an acetone sucrose extraction method (? 5) The control antigen was prepared in the same way from uninfected spleens and livers

Animals Outbred white Swiss mice aged three months weighing about 20 g were used in the experiments Similar mice aged about five to six weeks were used for the virus titrations

Cortisone treatment Cortone® acetate was used and the single dose was 0 8 m It was given subcutaneously every second day for a fortnight, starting on the day of virus inoculation This cortisone dose was chosen after experiments in which groups of adult outbred mice as used in the present experiments were treated with 0 4 mg 0 8 mg and 1 2 mg every second day *A single dose of 0 8 mg* was found to be the maximum tolerated by these animals by this method of administration All the animals were clinically affected by the drug after three to four injections Their pelts were ruffled their skin fragile with occasional ulcerations They were hypokinetic with a congested appearance

Methotrexate treatment Sodium methotrexate® was used in dilution with 2 per cent hydrogen carbonate solution It was administered subcutaneously every second day for a fortnight beginning on the day of the virus inoculation Preliminary experiments using 0 02 mg 0 04 mg and 0 08 mg every second day to adult outbred mice *showed that 0 02 mg repre ented the maximum tolerated dose by this method* of administration These animals were also here clinically affected b the drug after three to four injections

X ray irradiation The mice were irradiated (whole body) by being placed in small holes in a cylinder which rotated under the tube at a distance of 51 cm. The dose rate was 40 r per minute. The physical constants were 175 k 8 mA 0 5 mm Cu The dose was measured by a Victoreen Integron dosimeter After correction for penetration and back scatter the true dose was 430 r All the irradiated animals showed a transient loss of weight during the first week after the treatment. The irradiation was given on the same day as the virus inoculation

RESULTS

The Effect of Cortisone

Two groups each consisting of 30 adult mice were inoculated intracerebrally with 100 and 1 000 LD_{50} LCM virus respectively Both groups were treated with cortisone as described By the end of a fort night 18 and 17 mice from the first and second group respectively had died During the following weeks a number of the remainder died The survivors were followed by individual blood samples during the next six months Then they were killed and their blood spleens and kidneys were titrated for virus contents There were no investigations of antibody production The results of these *observations are listed in* Table 1 This table also contains the results of two groups of simi

TABLE 1

The Lethality and the Course of the Virus Titres in the Blood and Organs of Adult Outbred Swiss Mice Inoculated with LCM Virus and Treated with Cortisone or Methotrexate or Left Untreat I

Virus dose	Lethality (14 days)	Treatment	5 weeks after inoc Blood	8 weeks after inoc Blood	11 weeks after inoc Blood	16 weeks after inoc Blood	21 weeks after inoc Blood	6 months after inoc Blood	6 months after inoc Spleen	6 months after inoc Kidney
100 LD_{50}	18/30	Cortisone	4 $>10^{1 0}$§ 7 <10	3 $>10^{0 5}$ 8 <10	–	11 $<10^{0 3}$	11 $<10^{0 3}$	11 <10	11 <10	3 <10 8 $>10^{2 5}$
1000 LD_{50}	17/30	Cortisone	3 $>10^{1 0}$ 9 <10	11 $<10^{0 3}$	–	11 $<10^{0 3}$	11 $<10^{0 3}$	11 <10	11 <10	11 $>10^{3}$
100 LD_{50}	0/10	Methotrexate	10 $>10^{8}$	10 $<10^{5}$	10 $<10^{0 3}$	10 <10	10 $<10^{0 3}$	10 $<10^{0 8}$	10 <10	10 $>10^{3}$
1000 LD_{50}	0/10	Methotrexate	10 $>10^{6}$	10 $<10^{1 0}$	10 $<10^{0 3}$	10 <10	10 <10	10 <10	10 $<10^{0 3}$	1 <10 9 $>10^{2 3}$
100 LD_{50}	8/10	none	1 $10^{0 5}$ 1 <10	2 <10	2 <10	2 <10	2 <10	2 <10	2 <10	2 $>10^{4}$
1000 LD 0	7/10	none	3 <10	3 <10	3 <10	3 <10	3 <10	3 <10	3 <10	1 <10 2 $>10^{5}$

Number of dead within 14 days/number of inoculated mice

§ Number of mice virus titre

TABLE 2

Virus Titres and CF Titres in the Blood and Organs of Adult Outbred Swiss Mice Inoculated Intraperitoneally with Varying Doses of LCM Virus All the Animals are Treated with Cortisone The Animals were killed at the Time of Investigation

Virus dose	1 month after inoc		2½ months after inoc			4 months after inoc			15 months after inoc			
		Virus titre		Virus titres			Virus titres			Virus titres		
	CF	Blood	CF	Spleen	Kidney	CF	Spleen	Kidney	CF	Blood	Spleen	Kidney
3×10^{6} LD_{50}	64	N T §	64 64	N T N T	N T >10 5	64 64	N T N T	N T 101 s	32 32 32 16	N T N T Trace Trace	N T Trace N T N T	Trace N T 10 s 101 s
3×10^{4} LD_{50}	64	N T	128 128	N T Trace	>10 5 >10	64 64	N T N T	Trace >10 5	32	N T	N T	>10 5
3×10^{2} LD_{50}	64	N T	256 256	N T N T	>10 5 N T	16 32	N T N T	N T N T	32	N T	Trace	>10 5
3×10^{0} LD_{50}	<4	N T	<4 <4	N T N T	N T N T	<4 <4	N T N T	N T N T	<4 <4 <4 <4	N T N T N T N T	N T N T N T N T	Trace N T N T N T

Titration on a pool of blood from the whole group
§ N T = no trace

TABLE 3

Virus Titres and CF Titres in the Blood and Organs of Adult Outbred Swiss Mice Inoculated Intraperitoneally with Varying Doses of LCM Virus The Animals were Killed at the Time of Investigation

Virus dose	1 month after inoc		2 ½ months after inoc			4 months after inoc			15 months after inoc			
		Virus titre		Virus titres			Virus titres			Virus titres		
	CF	Blood	CF	Spleen	Kidney	CF	Spleen	Kidney	CF	Blood	Spleen	Kidney
3×10^{6} LD_{50}	64	NT§	64	NT	NT	64	$10^{1.3}$	>10 5	16	NT	Trace	NT
			64	NT	NT	32	Trace	NT	32	NT	NT	NT
			64	NT	NT	64	NT	NT	16	NT	Trace	NT
3×10^{4} LD_{50}	32	NT	64	NT	NT	64	NT	NT	16	NT	NT	NT
			64	NT	NT	32	NT	NT	16	NT	NT	NT
			128	Trace	>10 3	64	NT	NT	32	NT	NT	NT
3×10^{3} LD_{50}	64	NT	8	Trace	NT	64	NT	NT	32	NT	NT	NT
			64	Trace	NT	64	NT	NT	32	NT	NT	>10
			32	NT	NT	64	NT	NT	64	NT	NT	NT
3×10^{0} LD_{50}	8	NT	128	NT	NT	<4	NT	NT	<4	NT	NT	NT
			<4	NT	NT	64	$10^{1.3}$	NT	<4	NT	NT	NT
			<4	NT	NT	<4	NT	NT	<4	NT	NT	NT

* Titration on a pool of blood from the whole group

§ NT = no trace

larly inoculated mice which were left untreated Only two and three mice respectively survived (out of ten) and were followed

The results show a moderate decrease in the lethality rate in the cortisone-treated groups The viraemia was eliminated after five to eight weeks in most of the mice After six months the virus had been eliminated from the blood and spleens but in most of the animals—both those treated with cortisone and the controls—a high virus content still persisted in the kidneys None of these animals developed tolerance

In order to see whether tolerance could be induced in the adult cortisone treated mouse by means of large amounts of virus the following experiments were performed Groups of adult mice (10 mice per group) were inoculated intraperitoneally with 3 LD_{50} 3 × 10 LD_{50} 3 × 10^4 LD_{50} and 3 × 10^6 LD_{50} All the animals were treated with cortisone At intervals hereafter one to four animals per group were killed Their blood was titrated for CF antibodies and their organs for virus content The results of these observations are recorded in Table 2 A total of 26 mice were tested 14 died during the long observation period

For comparison the results of similarly inoculated groups of mice which were left untreated are recorded in Table 3

No distinct differences were found between the treated and the untreated groups as regards the virus elimination and the antibody formation No states of tolerance developed in the cortisone treated groups not even after the large virus dose In the treated groups four mice still had high virus content in the kidneys after 15 months There were similar findings in only one mouse in the untreated group

The Effect of Methotrexate

A series of experiments on similar lines to the cortisone experiments were performed using methotrexate

After intracerebral inoculation of 100 and 1 000 LD LCM virus into two groups of ten mice each the lethality in the animals treated with methotrexate was nil after a fortnight The results from these groups are also listed in Table 1 High viraemia was still found four weeks after the virus inoculation but it disappeared during the following weeks All mice investigated after six months had high virus content in the kidneys

After intraperitoneal inoculation of 3 LD_{50} 3 × 10 LD_{50} 3 × 10^4 LD_{50} and 3 × 10^6 LD_{50} (see Table 4) the CF titres were lower after one month than in the corresponding cortisone treated and untreated groups (Tables 2 and 3) The virus was eliminated at a slower rate from the spleens and the high renal virus content was found much more frequent in this experiment It was found in all investigated animals except in the group inoculated with the low virus dosage Only

TABLE 4

Virus Titres and CF Titres in the Blood and Organs of Adult Outbred Swiss Mice Inoculated Intraperitoneally with Varying Doses of LCM Virus. All the Animals are Treated with Methotrexate. The Animals were Killed at the Time of Investigation

Virus dose	1 month after inoc CF	1 month after inoc Virus titre Blood	2 ½ months after inoc CF	2 ½ months after inoc Virus titres Spleen	2 ½ months after inoc Virus titres Kidney	4 months after inoc CF	4 months after inoc Virus titres Spleen	4 months after inoc Virus titres Kidney	15 months after inoc CF	15 months after inoc Virus titres Blood	15 months after inoc Virus titres Spleen	15 months after inoc Virus titres Kidney
3×10^{6} ID	9	>10	<4	>10 5	$>10^{2.5}$	32	N T§	>10 5	<4	Trace	$10^{1.6}$	>10
			<4	N T	>10	<4	N T	>10 5	<4	N T	$10^{1.8}$	>10 5
3×10^{4} ID	<4	>10 5	32	10	$>10^{2.5}$	<4	N T	$>10^{2.5}$	16	N T	N T	>10 5
			8	N T	>10 5	<4	N T	$>10^{2.5}$	8	N T	N T	$10^{1.5}$
									16	N T	$10^{1.0}$	>10 5
									<4	>10 5	$>10^{2.5}$	$>10^{2}$
3×10^{2} ID_{50}	<4	>10 5	16	$10^{1.8}$	>10 5	16	10 3	$>10^{2.5}$	16	10	Trace	>10 5
			<4	$10^{1.0}$	>10 5	<4	N T	$>10^{2.5}$	32	10	N T	>10 5
									8	N T	N T	$10^{1.3}$
3×10^{0} ID_{50}	32	>10 5	<4	N T	N T	<4	N T	N T	<4	N T	N T	N T
			128	N T	N T	<4	N T	N T	<4	N T	$10^{1.0}$	N T
									16	N T	Trace	N T

Titration on a pool of blood from the whole group

§ N T = no trace

(nos 1 and 8 in the 100 LD_{50} group) no antibodies were found at any time despite high viraemia at the beginning of the observation period

DISCUSSION

In the present LCM virus/mouse system the intracerebral inoculation is the most sensitive means of estimating the lethal effect of the virus (cf LD_{50} determinations) In the present experiments in which the intracerebral route has been used a clear cut effect of the three immunosuppressive procedures on the lethality rate has been found (Tables 1 and 5) This effect is most pronounced in the methotrexate treatment where the lethality rate is nil Similarly low lethality rate has also been found in therapy with ATS or rabbit anti mouse IgG (6 13) In the present experiments no histopathological examinations were made It might be assumed that the lymphoid infiltrates in the meninges and choroid plexus—possibly provoked by the virus as localized immunological reactions of the host to the virus and considered to be the cause of death of the animals (3 23 24)—were also abolished to some extent by the therapy resulting in survival of the animals

Before the discussion on the development of tolerance the author s criteria for complete and incomplete tolerance will be stated These criteria are based on observations of thousands of tolerant animals The criteria for a complete tolerance in the present mouse/virus system are 1) The presence of a long lived content of virus in the animal as expressed by virus titres of $\gtreqless 10^{-5}$ in the blood 2) The absence of measurable amounts of CF antibodies titres <4 At no time has any immunological disease or wasting been found among mice fulfilling these criteria provided that the tolerance was induced by *inoculation of a sufficient virus dose within the first 18 hours of life* (11 27) The incompletely tolerant states in this system may take various forms 1 The virus titres in the blood are at virus carrier level (*i e* $\gtreqless 10^{-5}$) together with CF antibody formation 2 The virus titres are below virus carrier level without CF antibody formation 3 A combination of both 1 and 2 These incompletely tolerant states are usually transient and often result in a weakening of the animal (10 14)

In the present experiments no development of tolerance was found in the intracerebrally inoculated mice treated with cortisone or methotrexate The virus elimination was somewhat more delayed in the *methotrexate treated groups indicating a suppression of this* mechanism The phenomenon of maintained high virus titres in the kidneys was very pronounced in these groups even after six months This indicates that the virus eliminating mechanism did not function in the kidneys As this virus elimination is most probably mainly dependent on a cell mediated immune response of the host to LCM

virus (8 10 11 12) this would suggest the existence in the kidneys of an immune barrier to this immune response The same phenomenon has previously been found in the same virus/mouse system where the tolerant state has been abolished by adoptive immunization (8 26 27)

In the irradiated mice (Table 5) the virus elimination was also suppressed in most of the animals but antibodies were formed in quite high titres However in only two of the mice had the immune response been suppressed completely with the resultant development of a complete tolerance This occurred in mice nos 1 and 5 in the 100 LD_{50} group The latter had in addition a transient phase of antibody formation In mouse no 8 in the same group there was virus elimination without any humoral immune response This part of the immune response must have been suppressed while the other part—the cell mediated—was not *i e* an incompletely tolerant state presumably indicating a state of split tolerance This phenomenon has also been found in some mice inoculated at an age of 2 to 9 days with LCM virus (10) In several of the mice in these irradiated groups it was found that high virus titres and CF titres were present at the same time This presumably also indicated states of split tolerance

The experiments with intraperitoneal inoculation of varying virus doses (Tables 2 3 and 4) illustrate that large amounts of this virus strain did not harm the mice As can be seen in the untreated groups (Table 3) the virus was eliminated from the blood after four weeks and antibodies were formed at this time Only few mice continued to have high renal virus titres No states of tolerance were found in any of these untreated mice In the cortisone treated groups the virus was also eliminated from the blood after four weeks but the high renal (and to a lesser degree—splenic) virus titres were more pronounced The antibody titres were by and large at a higher level than in the untreated groups No states of tolerance were found in any of the mice in these groups

In the methotrexate treated groups high viraemia and low or no antibody titres were found after four weeks After 2½ months virus elimination was functioning in most of the mice This means that the virus eliminating process (*i e* the cell mediated immune response) had been suppressed up to this time The antibody titres were at a very low level indicating a simultaneous suppression of the humoral response The suppression persisted in only two mice resulting in a completely tolerant state after 15 months The phenomenon of the persisting high renal virus titres is found in almost all mice in these groups except for the low virus inoculated groups

The low virus dose of 3 LD_{50} had only a slight or no immunogenic effect in all experiments this may presumably be explained by little or no multiplication of the virus after this dose There was no great difference between the results in mice inoculated with the very varied

(nos 1 and 8 in the 100 LD_{50} group) no antibodies were found at any time despite high viraemia at the beginning of the observation period

DISCUSSION

In the present LCM virus/mouse system the intracerebral inoculation is the most sensitive means of estimating the lethal effect of the virus (cf LD_{50} determinations) In the present experiments in which the intracerebral route has been used a clear cut effect of the three immunosuppressive procedures on the lethality rate has been found (Tables 1 and 5) This effect is most pronounced in the methotrexate treatment where the lethality rate is nil Similarly low lethality rate has also been found in therapy with ALS or rabbit anti mouse IgG (6 13) *In the present experiments no histopathological examinations* were made It might be assumed that the lymphoid infiltrates in the meninges and choroid plexus—possibly provoked by the virus as localized immunological reactions of the host to the virus and considered to be the cause of death of the animals (3 23 24)—were also abolished to some extent by the therapy resulting in survival of the animals

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IMMUNOCHEMICAL STUDIES OF ORAL FUSOBACTERIA

3 Purification of a Group Reactive Precipitinogen

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Received 23 iv 69

The major precipitinogens found in the *Fusobacterium* strain F1 isolated in our laboratory have been reported earlier (3) Some of the characteristics of the precipitinogens were also described (3) One of the precipitating antigens provisionally termed Precipitinogen 2 was found to be present in all of 20 strains of oral fusobacteria studied This precipitinogen was heat labile and was destroyed by 45 per cent phenol by digestion with pepsin and pronase and by oxidation with periodate Apparently Precipitinogen 2 is a group specific antigen containing protein and possibly a carbohydrate component (3)

Attempts have been made to purify this substance Some of our experiments along with a purification procedure for Precipitinogen 2 will be reported in the present paper

MATERIALS AND METHODS

Strains The methods for *isolation and characterization* of fusobacteria have been described (3) Strain F1 was selected to serve as source of Precipitinogen 2 in the present study Extracts from this microorganism showed high titres of Precipitinogen 2

For mass cultivation the bacteria were grown in fluid Brain Heart Infusion broth (Difco) usually for 4 days and harvested by centrifugation After harvesting the organisms were washed twice with sterile distilled water and stored as a paste at —25 C until use.

Disruption of bacteria was achieved by 5 passages of washed bacteria through the X Press (1)

Rabbit immune sera were prepared as described in (3) Antisera against the type strain ATCC 10 953 or against the strain F 30 a isolated in our laboratory precipitated only line 2 in agar with most extracts from strain F 1 These antisera were used to follow the titres of Precipitinogen 2 during extraction and fractionation procedures Antiserum against strain F 1 and antisera against 8 additional strains of *Fusobacteria* were used to check for the presence of precipitating antigens other than Precipitinogen 2 in the fractions

Agar double diffusion tests were performed as previously described (3) Diffusion

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and precipitation took place at room temperature Whenever the objective of the test was to follow the agar precipitation titre during fractionation and purification processes two fold serial dilutions of the antigenic fraction were put into the peripheral wells and undiluted antiserum in the central well In these instances readings were made after 24 hours Otherwise readings were made after 1 2 and 3 days

The methods used for *ring test precipitation immunoelectrophoresis* and *paper electrophoresis* were those described in (3) Electrophoresis strips were stained with amido black only

For *gel filtration* with Sephadex G 25 G 50 G 100 and G 200 (AB Pharmacia Uppsala Sweden) columns were packed largely as described by *Flodin* (2) The ratio diameter height of the columns varied from 1 10 to 1 15 Columns of Sephadex G 25 Coarse used for desalting were calculated (5) to give complete separation of the active material and salts in the sample volume to be desalted Experiments to test the efficiency of the columns for desalting were also run

Columns for *ion exchange chromatography* were prepared from DEAE cellulose (DEAE SS Serva Heidelberg) The ratio diameter height of the columns varied from 1 5 to 1 10

Preparative ultra centrifugation was performed in a Beckman Spinco Model L ultracentrifuge in 10 ml tubes

The method employed for *ultrafiltration* was similar to that described in (6) except that a glass cylinder 2 5 × 120 cm equipped with a vacuum outlet was used instead of a desiccator The dialysis bag (Arthur Thomas Philadelphia) had an extended diameter of ¹ inch and was connected to a sample reservoir The volume of a solution could be reduced by more than 2a ml per hour at 4 C in this way

Concentration with Sephadex (2) was achieved by adding amounts of dry Sephadex G 25 Coarse calculated to give a two fold concentration After 15 minutes the active material was removed from the swollen gel by pressure filtration

Proteins were determined by the Folin Ciocalteau phenol method according to *Lowry et al* (4) with bovine serum albumin as standard

Neutral sugars were estimated by the sulphuric acid orcinol method (9) Since preliminary chromatographic examinations of acid hydrolysates of our crude extracts and fractions had indicated that glucose was the dominant neutral sugar present glucose was used as standard

Total free lipids were estimated largely as described by *Sperry* (8) *Fatty acid esters* were determined by the method described by *Snyder & Stephens* (7) Tripalmitin was used as standard

Total activity is defined as the precipitation titre in agar multiplied by the volume in ml

Specific activity is defined as the precipitation titre in agar divided by the Folin protein value expressed in mg/ml

Unless otherwise stated all fractionation procedures were carried out at room temperature

EXPERIMENTS AND RESULTS

A *Preliminary Experiments*

Some results from preliminary experiments which provided a foundation for a purification procedure will be reported in some detail

Extraction A number of experiments were carried out to determine optimal conditions for extraction of Precipitinogen 2 from microorganisms Disintegration of the bacteria prior to extraction invariably gave superior results The yield was increased as much as four fold by crushing the organisms prior to extraction The specific activity was the same with crushed and whole bacteria

Crushed microorganisms were extracted with different buffers at various temperatures Extraction was continued for 24 hours and the supernatant after centrifugation at 20 000 x *g* for 30 minutes was col

lected Extraction with 0 05 M phosphate buffer pH 7 4 at 4 C was found to give good results

Stability of extracts Crude buffer extracts could be stored for several days at 4 C without apparent loss of Precipitinogen 2 After a certain degree of purification had been achieved the material became very unstable however Clear solutions containing Precipitinogen 2 became opaque flocculent precipitates appeared and the precipitating ability disappeared or was greatly reduced These alterations were observed particularly when solutions of partially purified material were subjected to prolonged dialysis against tap or distilled water Concentration of purified or partially purified solutions under reduced pressure at temperatures well below 40 C resulted in a rapid and eventually complete loss of activity of Precipitinogen 2 Freezing and thawing had a similar effect Lyophilization of dialyzed solutions rendered the material nearly insoluble in aqueous media even at strongly alkaline pH The denaturation and inactivation appeared to be irreversible

Precipitinogen 2 could be at least partially protected from denaturation during purification procedures by including 0 001 M ethylene diaminetetraacetate (EDTA) and 0 005 M 2 mercaptoethanol (ME) in the buffer solutions used Partially purified solutions could be stored for several days at 4 C in this way without serious loss of serological activity

Fractional precipitation Precipitation with acid precipitation with varying concentrations of Rivanol (Hoechst, Frankfurt a M) and fractional precipitation with organic solvents at room temperature and at 4 C gave poor yields and little or no purification Precipitinogen 2 could however be precipitated in good yields from crude extracts or partially purified solutions with 3 volumes of acetone at −24 C Acetone precipitates were collected by rapid centrifugation in a refrigerated centrifuge dried *in vacuo* and ground to a fine powder Such powders could be washed with ethanol ether (2 1) at −24 C without apparent damage to the active material

Dialyzed and lyophilized crude buffer extracts from strain F1 contained 10–15 per cent of free lipids and approximately 10 per cent of fatty acid esters Some of the lipid material was not readily disposed of during later stages of the purification procedure Treatment with acetone and ethanol ether at −24 C not only served to remove free lipid but also removed a considerable amount of contaminating proteins The purification in this respect was almost three fold Equally good results were obtained when a suspension of crushed microorganisms in distilled water was subjected to the acetone and ethanol ether treatments at −24 C prior to extraction for 24 hours with 0 05 M phosphate buffer pH 7 4 The latter procedure had the additional advantage that it involved the handling of smaller volumes Five grams of bacteria treated in this manner did not contain measureable amounts of free lipid

Almost 80 per cent of Precipitinogen 2 was precipitated from crude extracts between 40 and 70 per cent saturation with ammonium sulphate and a three fold purification could be achieved Most of the Freeman type hapten (3) and the endotoxic lipopolysaccharide (3) were also found in the 40–70 per cent ammonium sulphate fraction The 40–50 50–60 and 60–70 per cent fractions were found to be similar with regard to both total activity and specific activity

Column chromatography Precipitinogen 2 could be adsorbed to columns of DEAE cellulose equilibrated with suitable buffers when partially purified extracts were applied to the colums The material was eluted in good yields by gradient or stepwise elution with increasing salt concentrations A column of DEAE cellulose equilibrated with 0 02 M phosphate buffer pH 6 3 containing 0 001 M EDTA and 0 005 M ME was found to give good results Unless the material applied to the columns had been sufficiently purified certain contaminants notably polysaccharide materials identifiable as Freeman type hapten and endotoxic lipopolysaccharide were eluted from the columns together with Precipitinogen 2

Ultracentrifugation and gel filtration Most of the endotoxic lipopolysaccharide could be removed prior to ion exchange chromatography by ultracentrifugation at 100 000 x *g* for 1 hour By this procedure the lipopolysaccharide was precipitated as a gelatinous material Gel filtration of the supernatant through columns of Sephadex G 200 separated Precipitinogen 2 from the Freeman type hapten and residual lipopolysaccharide fairly well Fractions from columns of Sephadex G 200 which gave only the line corresponding to Precipitinogen 2 in agar contained about 60 per cent of the activity of the sample applied to the column and the specific activity was about 5 times higher

Concentration and desalting Attempts to concentrate and desalt solutions of purified Precipitinogen 2 prior to lyophilization generally resulted in severe denaturation and loss of serological activity Even with ultrafiltration for 6 hours at 4 C considerable precipitation with concomitant loss of activity occurred Concentration with Sephadex G 25 Coarse gave the best results A four fold concentration could be obtained in two steps with 60–80 per cent yield of active soluble material The specific activity was unchanged

The time needed for desalting by dialysis could be shortened considerably by increasing the surface area per unit volume of solution and by providing for stirring inside and outside the dialysis bag However some precipitation and loss of activity still occurred On desalting by gel filtration on columns of Sephadex G 25 Coarse some of the active material tended to precipitate in the gel bed The recovery of active soluble material by this procedure was usually about 70 per cent The product obtained after freeze drying of material desalted

n this manner was white and fluffy soluble in aqueous media at neutral pH and had the same specific activity as before desalting

3 *Purification Procedure*

On the basis of the preliminary experiments a procedure for the purification of Precipitinogen 2 could be adopted It comprised the following steps

1) Preparation of an acetone powder from crushed microorganisms Further removal of lipids with ethanol ether (2 1)
2) Extraction of dried defatted microorganisms with 0 05 M phos phate buffer pH 7 4 at 4 C
3) *Fractional precipitation with ammonium sulphate* All buffers used after this step contained 0 005 M MF and 0 001 M EDTA
4) *Ultracentrifugation at 100 000 x g*
5) Gel filtration on Sephadex G 200
6) Ion exchange chromatography on DEAE cellulose at pH 6 3

A detailed account of the purification procedure will be presented by reporting the purification of one batch of Precipitinogen 2 Some details of the purification procedure are shown in Table 1

TABLE 1
Purification Steps for Precipitinogen 2

Purification steps	Volume ml	Agar precipitation titre serum F30a (reciprocal)	Recovery per cent	Protein mg/ml	Specific activity
Extract from crushed "defatted dried bacteria	210	8	100	4 96	1 6
Ammonium sulphate precipitate	40	32	76	12 50	2 7
Ultracentrifugation and gel filtration on G 200	100	8	48	0 57	14 0
Ion exchange chromatography	190	4	45	0 16	25 0

In this experiment 40 g (wet weight) of crushed microorganisms strain F1 were suspended in 50 ml of distilled water at 4 C This slurry was poured slowly into 3 volumes of acetone prechilled to -24 C and extracted with gentle stirring at this temperature for about 5 minutes The microorganisms were collected by rapid centrifugation in a re frigerated centrifuge at 4 C and dried *in vacuo* overnight The dried

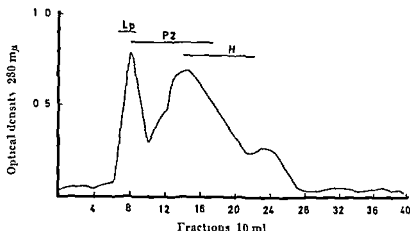


Fig 1

Purification of Precipitinogen 2 by gel filtration on Sephadex G 200 at pH 6 3
Lp = Endotoxic lipopolysaccharide
P2 = Precipitinogen 2
H = Freeman type hapten

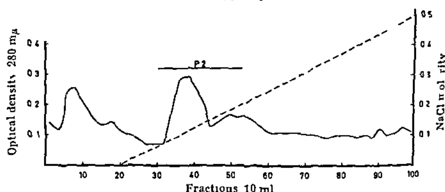


Fig 2

Purification of Precipitinogen 2 by column chromatography on DEAE cellulose at pH 7 3
P2 = Precipitinogen 2

material was ground to a fine powder and extracted for 10 minutes with 100 ml ethanol ether (2 1) at —24 C The defatted microorganisms were again dried in *vacuo* after rapid centrifugation The weight of the dried defatted microorganisms was approximately 6 g This powder was suspended in 200 ml of 0 05 M phosphate buffer pH 7 4 by the use of a Servall Omni Mixer at low speed for 1 hour Extraction was then allowed to proceed overnight at 4 C The mixture was then centrifuged at 25 000 x *g* for 30 minutes the bacterial residue was washed with 30 ml of the same buffer and the supernatants were combined The total volume of the extract was 210 ml It contained 0 7 mg of neutral sugars per ml The agar precipitation titre against an antiserum to strain F 30a was 8 and the specific activity was 1 6 To this

solution was added 50 g ammonium sulphate at room temperature When the salt had dissolved the solution was left at 4 C for 1 hour The precipitate after centrifugation at 12 000 x g for 30 minutes was discarded and another 67 g of ammonium sulphate was added to the supernatant with stirring The mixture was left at 4 C for 1 hour after the salt was dissolved and centrifuged as before The precipitate was dissolved in 0 02 M phosphate buffer pH 6 3 containing 0 001 M EDTA and 0 005 M ME and made up to 40 ml in the same buffer Approximately 75 per cent of the precipitating activity was recovered in this fraction and the specific activity was twice as high as in the original extract

This material was subjected to ultracentrifugation at 100 000 x g for 1 hour The supernatant was drawn off and the gelatinous deposits were discarded

Twenty ml of the supernatant fluid was applied to each of two columns 3 × 45 cm packed with Sephadex G 200 The columns had been equilibrated with 0 02 M phosphate buffer pH 6 3 containing 0 005 M of ME and 0 001 M of EDTA Elution was carried out with the same buffer The flow rate was approximately 20 ml per hour and 10 ml fractions were collected Some data from one of the Sephadex fractions are shown in Fig 1 A small amount of lipopolysaccharide material appeared in the eluate after 60 ml and could be found in the next two 10 ml fractions Precipitinogen 2 appeared in fractions 8 through 16 with a peak activity (ring test titre) in fraction number 10 The maximal activity of Precipitinogen 2 did not correspond to any of the three peaks of U V absorbing material observed Material precipitating a line in agar corresponding to the Freeman type hapten appeared from fraction number 14 Fractions number 9 through 12 from both columns precipitated only one line in agar against homologous and heterologous antisera These fractions were pooled They contained more than 40 per cent of the original activity of Precipitinogen 2 and the purification obtained by gel filtration was about five fold The ultraviolet absorption spectrum however showed a peak of absorbancy at 260 mμ indicating the presence of relatively large amounts of nucleoproteins in the fractions

After purification by gel filtration the material was applied directly to a column of DEAE cellulose 2 8 × 25 cm (Fig 2) The column had been equilibrated with the same buffer that was used for gel filtration The eluate was collected in 10 ml fractions After the material had entered the column 200 ml of the same buffer was passed through Some U V absorbing material without precipitating activity passed through the column in these fractions A linear gradient to 0 5 M NaCl in the starting buffer was subsequently used for elution of the column The active material was eluted almost quantitatively between 0 1 and 0 2 M NaCl No other precipitinogens could be detected in these fractions The maximal activity of Precipitinogen 2 corresponded to a

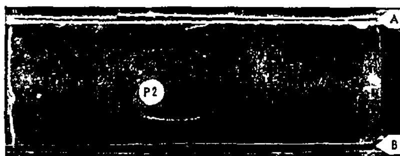


Fig 3

Immunoelectrophoresis of the purified Precipitinogen 2

P2 = Purified Precipitinogen 2 1 mg/ml in phosphate buffered saline
A = Antiserum to *Fusobacterium polymorphum* strain Fevl
B = Antiserum to strain Fl

single and fairly symmetrical peak When the NaCl molarity was raised above 0 6 materials with high optical density particulary at 260mμ appeared in the eluates but no active material was detected

The specific activity was increased about twofold by ion exchange chromatography The increase during the entire purification procedure not including the initial treatment of the bacteria with acetone and ethanol ether was about fifteen fold The content of neutral sugars in the unconcentrated material obtained from the DEAE cellulose column was too low to be measured accurately

The pooled active fractions were concentrated to approximately 40 ml with Sephadex G 25 Coarse desalted by passage on a column of the same material 5 × 45 cm and lyophilized The yield was 21 mg of a white fluffy material which gave clear solutions in water at neutral and alkaline pH in concentrations up to a least 2 mg/ml

The ultraviolet absorption spectrum of a 0 2 per cent solution of the material in buffered saline showed no peak or shoulder at 260mμ

Only one line was formed in agar double diffusion tests and immunoelectrophoresis experiments when a solution containing 1 mg/ml was used as antigen against homologous and heterologous antisera (Fig 3) When samples containing approximately 1 0 mg of protein (*Folin* value) were passed through columns 1 × 25 cm of Sephadex G 25 G 100 and G 200 equilibrated with the buffer used previously for gel filtration the material was eluted as a single symmetrical peak

On paper electrophoresis in buffers with pH 6 6 (phosphate buffer I = 0 1) pH 8 6 (veronal buffer I = 0 1) and pH 8 9 (High Resolution Buffer LKB Stockholm) the material moved slowly and as a single band towards the anode

DISCUSSION

Although the agar precipitation titre utilizing two fold serial dilutions gave reproducible results admittedly this method can only give rough

estimates of the total activity and specific activity of an extract and more subtle differences between two fractions can not be detected The estimates of recovery and specific activity presented in Table 1 therefore can only be regarded as approximates Ring test titres of Precipitinogen 2 were usually 4 times higher than agar precipitation titres and more exact estimates of the serological activity could be obtained However since non specific precipitation was produced with several of our antisera at pH 6 3 dialysis of such fractions would be necessary The ring test with appropriate controls therefore was used only for some of the introductory experiments

The instability in solution of Precipitinogen 2 has presented considerable difficulties during purification experiments It appeared from introductory experiments that several mechanisms might be responsible for the denaturation and inactivation of Precipitinogen 2 No attempt has been made to study this problem further nor has the possible stabilizing effect of EDTA and ME when used separately been studied Examination of residual activity of Precipitinogen 2 in the fractions which have been discarded during the purification process suggests that very little of the activity is lost through denaturation by the methods employed

The extraction of the microorganisms first with acetone and then with ethanol ether (2 1) appeared to be very effective in removing free lipids The extract obtained from defatted microorganisms also contained less contaminating protein than extracts obtained by direct extraction of bacteria If this initial step is taken into account the purification of Precipitinogen 2 achieved through the entire process amounts to about 75 fold

Precipitation with ammonium sulphate was studied in some detail but no further purification than that reported could be achieved without considerable loss of active material

Ultracentrifugation at 100 000 x *g* for 1 hour was a simple and convenient method for removing high molecular contaminants such as lipopolysaccharide complexes The loss of Precipitinogen 2 by this procedure was negligible

Gel filtration on Sephadex G 200 gave somewhat better results when smaller samples were applied to the columns Increasing the column volume had the same effect The procedure described has proved to be convenient for a standard preparative technique A considerable purification was obtained Furthermore the salt concentration in the fractions containing Precipitinogen 2 was adjusted to a level which made it possible to adsorb the material to colums of DEAE cellulose directly without including a dialysis step

The recovery from the DEAE cellulose columns was over 90 per cent and the specific activity was increased about two fold There appeared to be no indication that contaminating substances were eluted from the ion exchange columns along with Precipitinogen 2

The losses of active material during the final concentration and desalting procedures were considerable However these procedures with subsequent lyophilization resulted in a purified serologically active product which could be stored for months for the purpose of serological investigations

The techniques employed resulted in a product which was free from contaminating precipitinogens It also was free from other contaminants detectable by the gel filtration and electrophoresis experiments performed Additional tests for the homogeneity and purity of the product have been difficult to perform particularly because of the instability of Precipitinogen 2 in solution

Investigations on some chemical and serological properties of Precipitinogen 2 are in progress

SUMMARY

A method for the purification of a group specific precipitinogen from oral fusobacteria has been described The purification procedure involves the following steps

1 Extraction from crushed defatted and dried microorganisms
2 Fractional precipitation with ammonium sulphate
3 Ultracentrifugation and gel filtration
4 Ion exchange chromatography

The product obtained was homogeneous in agar precipitation immunoelectrophoresis gel filtration and paper electrophoresis experiments

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IMMUNOCHEMICAL STUDIES OF ORAL FUSOBACTERIA

4 *Some Chemical Properties of a Group Reactive Precipitinogen*

By

TORE KRISTOFFERSEN

Received 23 iv 69

In a previous communication the isolation of a group reactive precipitinogen from a strain of *Fusobacterium* was described (15) The product provisionally named Precipitinogen 2 was found to be homogeneous in paper electrophoresis gel filtration agar precipitation and immune electrophoresis experiments The material was very unstable in solution and was readily denatured during purification procedures particularly during the final concentration and desalting processes

The present paper deals with some chemical properties of the purified Precipitinogen 2

MATERIALS

Preparation no 1 The batch of Precipitinogen 2 for which the purification procedure was described in detail in (15) The lyophilized material was white light and gave clear solutions in aqueous media at neutral pH

Preparations nos 2 and 3 Precipitinogen 2 which had been prepared exactly as described in (15) except that the final concentration and desalting was achieved through ultrafiltration and dialysis This gave a considerably higher yield of freeze dried material although most of the serological activity was lost in these final steps The freeze dried product was only slightly soluble in aqueous media even at strongly alkaline pH The specific activity (15) of these preparations prior to the final concentration and desalting procedures was the same as with Preparation no 1

The source of all antigen preparations was strain F1

Rabbit antiserum against whole bacteria strain F1 was prepared as reported earlier (13) The undiluted antiserum was mixed with an equal volume of 0 2M 2 mercaptoethanol After one hour at room temperature the mixture was dialyzed for 4 hours against 0 0 M iod acetamide and then against buffered saline over night

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The quantitative amino acid analysis was performed by Mr *Jens Bøe* Department of clinical Biochemistry University of Bergen School of Medicine The thin layer chromatography for lipids was done by Mrs *Inger Grundt* of the same department The author is indebted to these persons and to the head of the department Professor Karl Closs M D Ph D for their help and advice

These investigations have been supported by grants from *L Meltzers Høyskolefond* and from the *Norwegian Research Council for Science and the Humanities*

DISCUSSION

The qualitative analyses of the three preparations showed very good agreement. The components identified by the paper chromatographic methods used were 14 amino acids, the sugars glucose and xylose and a small lipid component.

Experiments reported in a previous communication (13) showed that the precipitating ability of Precipitinogen 2 was destroyed by proteolytic enzymes and by periodate oxidation, indicating the presence of both protein and carbohydrate. The results of paper electrophoresis experiments with and without borate in the electrophoresis buffers strongly suggest that protein and carbohydrate are linked together (10). This assumption was corroborated by the demonstration of both glucose and xylose in a specific immune precipitate. Rabbit IgG contains mannose and galactose roughly in the ratio 2 : 1 and fucose but neither glucose nor xylose (6). Since a protein component was found to be essential for the precipitating ability of Precipitinogen 2 (13), the glucose and xylose found in the immune precipitate most likely are linked to this protein. It appears unlikely that the glucose and xylose found in the immune precipitate should be parts of a contaminating precipitating antigen. No indication of the presence of such contaminants has been found (15).

The nitrogen, hexose, pentose and fatty acid esters found in preparation no. 2 account for some 95 per cent of the material without correction for water uptake during hydrolysis. The analysis of preparation no. 3 gave less satisfactory results. Both nitrogen and sugar values were lower than in preparation no. 2, whereas the contents of lipid and phosphorus were somewhat higher.

Variations in the lipid content in different batches of purified antigens form Gram-negative bacteria are frequently reported (12, 19). The somewhat divergent results of the quantitative analyses of the two preparations may partially be due to variations in the lipid contents not detectable by the hydroxamic acid assay as applied in the present study. The small amounts of material available for analysis have prevented further studies of the lipid component. Pilot experiments have indicated the presence of phospholipids along with other lipid classes, however. The higher phosphorus content in preparation no. 3 might be due to a higher content of phospholipids.

The Winzler orcinol assay gave very reproducible results and is believed to give reliable estimates of neutral sugars in glycoproteins even in the presence of small amounts of tryptophan (17). Other substances known to interfere in this assay were not detected.

It is difficult to ascertain which of the two reactions for pentoses produced the most accurate results. Since no heptose was found in Precipitinogen 2 and since non-carbohydrates are considered to in

terfere less in the cysteine sulphuric acid reaction (11 4) the values from the latter assay have been listed in Table 1

In addition to the amino acids identified on the paper chromatograms the automatic amino acid analyzer established the presence of phenylalanine histidine and trace amounts of methionine and separated isoleucine and leucine Several amino acids are liable to be decomposed during acid hydrolysis Tryptophan is very readily destroyed but losses may be expected also for cysteine cystine and tyrosine especially in the presence of carbohydrate (5) Since the preparation used for amino acid analysis gave a completely clear solution in 0 1N NaOH tryptophan could be estimated by direct spectrophotometry The absence in chromatograms of all hydrolysates of cystine cysteine and tyrosine did not encourage special efforts to possibly identify and quantitate these amino acids

No trace of diaminopimelic acid was found in any of the three preparations of Precipitinogen 2 This observation may be of some interest in view of the significance and almost ubiquitous occurrence of this amino acid in Gram negative bacteria (20) Studies by *Baird Parker* (2) as well as in our laboratory (14) have shown that diaminopimelic acid is present in *Fusobacterium* including strain F1

In the Rondle and Morgan assay for amino sugars a faint red colour was produced by both preparations analyzed Since no amino sugars were detected on paper or column chromatography of acid hydrolysates this colour was regarded as non specific Mixtures of amino acids particularly lysine with neutral sugars are known to give red colours in the Elson Morgan assay (8)

In view of the strong indications of a linkage between carbohydrate and protein in Precipitinogen 2 the failure to detect amino sugars is a matter of considerable interest In most known instances a carbohydrate protein linkage involves an amino sugar usually an acetyl D hexosamine (18) Few exceptions are known However there is strong evidence that other types of linkages than those involving amino sugars are possible Thus the findings of *Lindahl & Roden* (16) indicate that a glycosidic linkage between xylose and the hydroxyl group of serine constitues the carbohydrate peptide linkage in heparin A similar linkage may be present in chondroitin 4 sulphate (9) Serine and xylose were invariably found in our preparations However no attempt has been made to elucidate the nature of the binding of carbohydrate to protein in Precipitinogen 2

SUMMARY

Some chemical properties of a group reactive precipitinogen from the *Fusobacterium* strain F1 have been investigated One preparation contained approximately 85 per cent protein including 15 amino acids a carbohydrate component consisting of 0 1 per cent glucose and 0 7 per

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SUMMARY

Some chemical properties of a group reactive precipitinogen from the *Fusobacterium* strain F1 have been *investigated* One preparation contained approximately 8› per cent protein including 15 amino acids a carbohydrate component consisting of 9 1 per cent glucose and 0 7 per

cent xylose and a small amount of lipid Other preparations contained the same components There was however considerable variations in the relative quantities of the components between different preparations

Though no amino sugars were found the carbohydrate and protein appeared to be linked together The nature of the binding is not known

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PRODUCTION OF ANTIBIOTICS BY *EPIDERMOPHYTON FLOCCOSUM*

4 *The Antibacterial Activity of the 'Epidermophyton factor (EPF) Compared with that of some Steroid Antibiotics*

By

ALF WALLERSTRÖM

Received 13 v 69

Previous publications (*Wallerström* 1967 1968 1969) were concerned with the course of formation the antibacterial spectrum and some other properties of an antibiotic produced by a skin pathogenic fungus *Epidermophyton floccosum* (the Epidermophyton factor below call ed EPF) The antibiotic proved to have a characteristic antibacterial spectrum differentiating between *inter alia* corynebacteria and *Listeria*, and between staphylococci and streptococci In this and other respects EPF resembled fucidin and in the first survey (*Wallerström* 1967) it was found that EPF resistant staphylococcal strains were resistant also to the latter antibiotic

Fucidin belongs to a group of steroid antibiotics chemically characterized as unsaturated monobasic carboxylic acids with a proto lanostane skeleton probably all related to the sterol triterpene family (*Stewart* 1964) Antibiotics of this group have similar antibacterial spectra and cross resistance has been found between several of them (*Burton & Abraham* 1951) *Barber & Waterworth* 1962)

This paper concerns a comparison of the antibacterial spectrum and some other properties of EPF and the above mentioned group of steroid antibiotics As representatives of this group those that have been most thoroughly investigated were selected namely helvolic acid (fumigacin) cephalosporin P_1 and fucidin

MATERIAL AND METHODS

Antibiotics Samples of EPF were prepared from ether extracts of filtrates from submerged cultures of *Epidermophyton floccosum* as described earlier (*Wallerström* 1969) In order to eliminate penicillin which may be produced by *Epidermophyton* strains Difco Bactopenase was added to the filtrates before extraction Samples of steroid antibiotics were kindly supplied by Professor E P Abraham Oxford England and by Dr W O Godtfredsen Løvens Kemiske Fabrik Ballerup Denmark

Bacterial strains In studies on the antibacterial spectrum of EPF and steroid antibiotics 27 strains belonging to 12 different bacterial species (Table 1) were

used Most strains had recently been isolated from routine laboratory specimens as for β streptococci other than group A *Corynebacterium diphtheriae* and *Listeria monocytogenes* older or freeze dried strains were used *Neisseria pharyngis* and *N catarrhalis* were defined as described earlier (*Wallerström* 1967) —In addition a number of strains of micrococci and staphylococci that had been collected from skin lesions due to *F floccosum* or from healthy or yeast infected individuals in a previous study (*Wallerstrom* 1968) were studied for their susceptibility to EPF and fucidin only a few strains of EPF resistant staphylococci found in nose and throat swabs (*Wallerström* 1967) were tested in the same way

Media In most tests for antibiotic activity on solid media Oxoid Diagnostic Sensitivity Test Agar (DST agar) was used Streptococci pneumococci *Neisseria pharyngis* and *N catarrhalis* were tested on plates containing Oxoid Blood Agar Base No 2 and 4 per cent horse blood *N meningitidis* on hematin agar prepared from the same base and 7 per cent heated horse blood *N gonorrhoeae* was tested on a special medium containing horse blood and ascitic fluid (*Reyn et al* 1963) The liquid medium used in most experiments was Oxoid Nutrient Broth No 2 and in some experiments concerning increase of resistance of staphylococci and coryne bacteria to EPF or fucidin Oxoid Tryptone Soya Broth

Gradient plates 15 ml of DST agar containing 40 U/ml of EPF was poured into a slanting petri dish with a diameter of 85 mm and allowed to solidify An upper layer of 15 ml of DST agar containing no antibiotic was added the dish now being horizontal The maximal thickness of each layer was 6 mm The plates were used for experiments as soon as the top layer had solidified

Gel filtration Ether extracts of EPF used *inter alia* in experiments concerning antibacterial spectrum and comparison with steroid antibiotics were partially purified by gel filtration A Sephadex G 10 column K 15/90 measuring 15 × 790 mm (bed volume approximately 125 ml) was used with distilled water as eluent The EPF extracts were applied in volumes of 5–15 ml The eluent was run at a rate of 15 ml per h and collected in 10 minute fractions Fractions containing EPF as identified by their antibacterial activity in the agar diffusion test were pooled and concentrated by pervaporation at room temperature

In vitro increase of resistance of bacteria to EPF and fucidin Broth cultures of strains of *Staph aureus* one of which was the standard test strain used in a previous investigation (*Wallerstrom* 1969) were started from single colonies and used for inoculation of tubes with 2 ml broth containing 10 U/ml of EPF The first inoculum used was heavy 0 5 ml of a 20 h culture The bacteria were subcultured still once or twice on media with this concentration of EPF tubes containing 20 U/ml of EPF were then inoculated The inoculum of the subcultures varied between 0 04–0 15 ml depending on the density of the growth the incubation time was 20 h but when growth was poor the incubation time was extended to 48–72 h An analogous procedure was used for fucidin the amount of fucidin in the medium was successively increased from 2 to 100 mcg per ml in 3 steps

3 strains of *Corynebact diphtheriae* first had to be cultivated on media with a low concentration of EPF 2 U/ml subpassages were then made on media in which the concentration of EPF was increased in several steps to a final value of 15 U/ml —Two strains of *Corynebact spp* on the other side could be cultivated directly on media containing 15 U/ml of EPF

The susceptibility to antibiotics was tested by the agar diffusion test The supply of helvolic acid and cephalosporin P_1 was sparse therefore in experiments where the susceptibility of the bacteria to EPF was compared with that of steroid antibiotics paper discs were prepared. The discs had a diameter of 6 mm (manuf Schleicher & Schuell) and were imbibed with 0 01–0 04 ml of the antibiotic solution and dried As the antibiotics were only slightly soluble in water but soluble in acetone the latter solvent was used The amount of antibiotic in the discs was 0 35 –2 mcg of fucidin 0 75–7 5 mcg of cephalosporin P_1 1 75–14 mcg of helvolic acid and 0 5–15 U of EPF (discs of four different strengths were made for each antibiotic) Discs giving identical or near identical zones with the standard strain of *Staph aureus* were chosen for the comparisons but each bacterial strain was tested with at least two different discs for each antibiotic The activity of the discs was checked every day against the standard strain of *Staph aureus*

A larger number of strains of staphylococci and micrococci were tested for their susceptibility to EPF and fucidin The tests were performed with agar cup technique on flood plates (*Wallerstrom* 1967) a sample of EPF containing 120 U per

ml was used The susceptibility to fucidin was tested with commercial discs containing 50 mcg of the antibiotic Some strains were tested also for their susceptibility to other antibiotics with discs from the same manufacturer (AB Biodisk, Stockholm 60 Sweden)

Testing of bactericidal/bacteriostatic activity A series of tubes containing different amounts of EPF (0 2–200 U/ml) were inoculated with approximately 10^3 bacteria per ml In this and the following experiments the inoculum consisted of staphylococci from a 20 h broth culture which had been started from a single colony the bacteria had been washed and resuspended in phosphate buffer pH 7 2 and the suspension treated for 5 minut s in a MSE Mullard ultrasonic disintegrator (frequency about 20 kilocycl s/sec) Viable counts showed that the treatment produced an approximately 30 per cent increase of the number of colonies growing from the suspension Treatment for longer periods did not produce any further increase in the number After incubation at 37 C for 24 h the tubes were tested for viable organisms by spreading a standard inoculum over the surface of two agar plates The plates were afterwards incubated at 37 C for 24 h. The lowest concentration at which no increase of turbidity occurred in the tubes was considered the bacteriostatic endpoint (minimal inhibitory concentration m i c) The lowest concentration at which no viable organisms were found on plating was considered the bactericidal end point (minimal bactericidal concentration m b c) In some experiments the incubation of the tubes was prolonged and viable counts were made after 2 3 and 4 days

In another experiment two series of tubes with 2 ml nutrient broth containing 5 20 and 40 U/ml of EPF respectively were inoculated with approximately 1 5 × 10^3 bacteria/ml from a 20 h broth culture of *Staph aureus* and incubated at 37 C. Surface viable counts were made after incubation for 2 4 and 24 h Each count was made by diluting samples 1 10 and 1 100 and spreading 0 1 ml of each on agar plates in duplicate

The number of EPF resistant cells in a population of staphylococci was tested by inoculating EPF containing solid media with predetermined numbers of *Staph aureus* from a 20 h broth culture. The inocula were prepared by suspending the bacteria in a volume of phosphate buffer corresponding to that of the original broth culture or in one tenth of this volume Each plate received 0 1 or 0 5 ml of the bacterial suspension The surface of the plate was about 60 cm^2 The number of bacteria in each suspension was checked by diluting it 1 10^5 1 10^6 and 1 10^7 and spreading 0 1 ml of each dilution on antibiotic free agar plates The number of EPF resistant mutants was estimated by counting and comparing the number of colonies that had grown on agar plates with and without EPF after incubation at 37 C for 48 h

RESULTS

The Antibacterial Spectrum of Steroid Antibiotics Compared with That of EPF

A number of bacterial strains were tested by the agar diffusion test for their susceptibility to EPF helvolic acid cephalosporin P_1 and fucidin The results of the tests are given in Table I

It is clear from the table that the antibacterial spectrum of EPF resembled that of cephalosporin P_1 and fucidin Helvolic acid was found to differ from the other substances in several respects being relatively more active against β hemolytic streptococci pneumococci and Neisseriae with the possible exception of N gonorrhoeae

The quantity of fucidin in the discs necessary to achieve a 24–25 mm inhibition zone with staphylococci was about half that of cephalosporin P_1 and one tenth of that of helvolic acid

In an earlier investigation (Wallerstrom 1968) a selected material of 390 strains of staphylococci and micrococci were tested for their

TABLE 1

Antibacterial Spectra of Some Steroid Antibiotics and of EPF Inhibition Zones (in mm) in the Agar Diffusion Test

	No of strains	Helvol acid 7 mcg disc.	Cephalosp P$_1$ 1 5 mcg disc	Fucidin 0 7 mcg disc	EPF 5 U disc.
Staphylococcus aureus (EPF susc test strain)	1	25	24	24	24
Staphylococcus aureus (EPF resistant trains)	4	0	0	0	0
α streptococci	2	0	0	0	0
β streptococci group A	1	23	0	0	0
β streptococci group A	2		±	0	0
β streptococci group H	1	21	0	0	0
β streptococci group L	1	25	0	0	0
Enterococci	1	±	0	0	±
Pneumococci	1	18	0	0	0
Corynebact dipht gravis	1			27	27
Corynebact dipht intermed	1			25	25
Corynebact dipht mitis	1	31	27	23	23
Corynebacterium spp					
strain 1	1	13	11 5	12 5	10
strain 2	1		23	16	15
strain 3	1	30		24	18
Listeria monocytogenes	1	±	0	0	0
Neisseria pharyngis	1	19 5	0	0	0
Neisseria catarrhalis	1	14 5	±	±	±
Neisseria meningitidis					
strain 1	1	18	9 5	10 5	10
strain 2	1	24		15	14
Neisseria gonorrhoeae	2	0–±	0	0	0

susceptibility to EPF and penicillin These strains were now tested against fucidin All EPF sensitive strains (301) proved sensitive to fucidin and the EPF resistant strains (89) were resistant to fucidin Strains whose growth was inhibited in large zones by fucidin (more than 35 mm in diameter with commercial discs containing 50 mcg of the antibiotic) also showed large inhibition zones when tested with EPF (mean value of zone diameter 32 7 mm)

In Vitro Increase of Resistance of Bacterial Strains to EPF and to Fucidin

In experiments with 9 originally EPF sensitive strains of *Staph aureus* the strains were found to grow well in the presence of successively increasing concentrations of EPF when the inoculum used ... more than 10^7 bacteria per ml) After 4–7 subcultures in ... up to 20 U/ml of EPF no zones occurred in the agar ... In another series of experiments the resistance of ... fucidin was increased in the same way by sub

culturing the strains on media containing up to 100 mcg per ml of fucidin

Three strains of *Corynebact diphteriae* and two strains of *Coryne bacterium spp* were propagated in the way described above on media containing successively increasing amounts of EPF eventually 15 U/ml After having been subcultured twice on media with the last mentioned concentration no inhibition zones occurred with the strains in the agar plate diffusion test with EPF whereas the sensitive parent strains showed zones which were 25-27 mm in diameter

In another experiment staphylococcal strains were cultured over night in liquid media containing a low concentration of EPF (1-2 U/ml) The inhibition zones in subsequent agar diffusion tests dimin ished and a number of small colonies appeared in the zones Such colonies were tested for their susceptibility to EPF in the tube dilution test They were found to have a m i c of 225 U/ml whereas the m i c of the parent strains was 0 2-0 5 U/ml The corresponding zones in the agar diffusion test had a diameter of 15 and 31 mm respectively

Experiments were made where gradient plates containing 40 U/ml of EPF in the bottom layer were flooded with staphylococci from two broth cultures that had been started from single colonies Each plate received approximately 10^7 bacteria After incubation for 48 h 5 colo nies which had grown on different gradient levels of each plate were isolated and tested for their susceptibility to EPF by the agar diffusion test The inhibition zones ranged between 17 and 20 mm in diameter and were thus of the same order of magnitude The inhibition zones of the parent strains were 31 mm

Demonstration of Cross Resistance between EPF and Steroid Antibiotics

It was found that staphylococci which were able to grow on media containing EPF had a decreased susceptibility to fucidin and *vice versa* (Table 2)

Cross resistance has been demonstrated between fucidin and cepha losporin P_1 (*Barber & Waterworth* 1962) and between the latter antibiotic and helvolic acid (*Burton & Abraham* 1951) For this reason some of the staphylococcal strains that had increased their resistance to EPF and fucidin were tested for their susceptibility to cephalosporin P_1 and helvolic acid as were their non resistant parent strain Cross resistance was found between all of the three steroid antibiotics and between these antibiotics and EPF (Table 3)

The susceptibility of the bacteria to tetracycline was tested since some EPF resistant strains of staphylococci encountered in an earlier investigation (*Wallerström* 1967) were resistant also to this antibio tic In the present study the development of resistance to EPF and steroid antibiotics was not found to influence on the susceptibility of the bacteria to tetracycline

TABLE 1

Antibacterial Spectra of Some Steroid Antibiotics and of EPF Inhibition Zones (in mm) in the Agar Diffusion Test

	No of strains	Helvol. acid 7 mcg disc.	Cephalosp P_1 1.5 mcg disc	Fucidin 0.7 mcg disc.	EPF 5 U disc.
Staphylococcus aureus (EPF susc test strain)	1	25	24	24	24
Staphylococcus aureus (EPF resistant strains)	4	0	0	0	0
α streptococci	2	0	0	0	0
β streptococci group A	1	23	0	0	0
β streptococci group A	2		±	0	0
β strepto cocci group H	1	21	0	0	0
β streptococci group L	1	25	0	0	0
Enterococci	1	±	0	0	±
Pneumococci	1	18	0	0	0
Corynebact dipht gravis	1			27	27
Corynebact dipht intermed	1			25	25
Corynebact dipht mitis	1	31	27	23	23
Corynebacterium spp					
strain 1	1	13	11.5	12.5	10
strain 2	1		23	16	15
strain 3	1	30		24	18
Listeria monocytogenes	1	±	0	0	0
Neisseria pharyngis	1	19.5	0	0	0
Neisseria catarrhalis	1	14.5	±	±	±
Neisseria meningitidis					
strain 1	1	18	9.5	10.5	10
strain 2	1	24		15	14
Neisseria gonorrhoeae	2	0–±	0	0	0

susceptibility to EPF and penicillin These strains were now tested against fucidin All EPF sensitive strains (301) proved sensitive to fucidin and the EPF resistant strains (89) were resistant to fucidin Strains whose growth was inhibited in large zones by fucidin (more than 35 mm in diameter with commercial discs containing 50 mcg of the antibiotic) also showed large inhibition zones when tested with EPF (mean value of zone diameter 32.7 mm)

In Vitro Increase of Resistance of Bacterial Strains to EPF and to Fucidin

In experiments with 9 originally EPF sensitive strains of *Staph aureus* the strains were found to grow well in the presence of successively increasing concentrations of EPF when the inoculum used was large (more than 10^7 bacteria per ml) After 4–7 subcultures in media containing up to 20 U/ml of EPF no zones occurred in the agar plate diffusion test In another series of experiments the resistance of the staphylococci to fucidin was increased in the same way by sub

TABLE 3

Effect of Culturing Staphylococci in Media Containing EPF or Fucidin Increase in Resistance to EPF and Steroid Antibiotics Agar Plate Diffusion Test

Staph strain labelled	Original zone diameter (in mm)					Zone diameter after growth in media containing EPF (in mm)					Zone diameter after growth in media containing fucidin (in mm)				
	Helvolic acid	Cephalosporin P_1	Fucidin	EPF	Tetracycl (control)	Helvolic acid	Cephalosporin P_1	Fucidin	EPF	Tetracycl (control)	Helvolic acid	Cephalosporin P_1	Fucidin	FPF	Tetracycl (control)
I	21	22	26	22	31	0	0	13	(+)	33	(+)	0	0	0	30
II	20	23	26	23	13	0	0	0	0	15	0	0	0	0	15
III	20	22		22	29	0	12	12	0	30	0	0	0	0	30
IV	21	22	25	20	28	0	0	0	0	29	0	0	0	0	27 5
V	22	21 5	25	22	29	0	0	0	0	30	0	0	0	0	28
VI	22	5	25	23	27 5	0	0	0	0	30	0	0	0	0	28

TABLE 2

Effect of Culturing Staphylococci in Media Containing EPF or Fucidin Mutual Increase of Resistance to Both Antibiotics Agar Plate Diffusion Test

Staph strain labelled	Original zone diameter (in mm)		Zone diameter after growth in media containing EPF (in mm)		Zone diameter after growth in media containing fucidin (in mm)	
	EPF 120 U/ml in cup	Fucidin 50 mcg disc	EPF 120 U/ml in cup	Fucidin 50 mcg disc	EPF 120 U/ml in cup	Fucidin 50 mcg disc
I	24	35	0	24	0	17
II	25	35	0	17	0	21
III	25	34	0	21	0	0
IV	24	33	0	13	0	10
V	25	35	0	20	0	±
VI	27	36	0	11	0	15
VII	29	39	0	11	0	13
VIII	25	35	0	19	0	20
IX	23	34	0	26	0	13

The Number of EPF Resistant Mutants in a Staphylococcal Population

Solid media containing EPF were inoculated with predetermined numbers of bacteria from 20 h broth cultures of *Staph aureus* It was found that suspensions with the same number of bacteria as the original broth culture (in three experiments 3 6 $\times$ 10^9 4 2 $\times$ 10^9 and 2 8 $\times$ 10^9 bacteria per ml) in most cases gave rise to no growth or only scattered colonies 0 1 ml of suspensions with the tenfold number of bacteria per ml resulted in growth of 29 3 5 and 16 5 staphylococcal colonies When tested in the agar diffusion test all colonies were found to have a lowered susceptibility to EPF the m i c determined in 15 colonies from a 20 U/ml plate ranged from 3 to 24 U/ml of EPF The number of resistant mutants in the staphylococcal populations was estimated from these results at 1 out of 10^7–10^9

Mode of Action of EPF on Bacteria (Bactericidal/Bacteriostatic Activity)

The standard test strain of *Staph aureus* was inoculated into a series of broth tubes containing 0 2–200 U/ml of EPF The inoculum used was approximately 10^3 bacteria per ml The m i c of the strain was 0 5 U/ml and the m b c 15 U/ml When the incubation of the tubes was extented to 3 or 4 days no viable bacteria were recovered from tubes containing more than 3 5 U/ml of EPF

When broth containing 5 20 or 40 U/ml of EPF was inoculated with approximately 1 5 $\times$ 10^4 bacteria per ml the number of bacteria growing from the culture persisted at the level of 1–3 $\times$ 10^4 after 2 and 6 h

TABLE 3

Effect of Culturing Staphylococci in Media Containing EPF or Fucidin Increase in Resistance to EPF and Steroid Antibiotics Agar Plate Diffusion Test

Staph strain labelled	Original zone diameter (in mm)					Zone diameter after growth in media containing EPF (in mm)					Zone diameter after growth in media containing fucidin (in mm)				
	Helvolic acid	Cephalosporin P_1	Fucidin	EPF	Tetracycl (control)	Helvolic acid	Cephalosporin P_1	Fucidin	EPF	Tetracycl (control)	Helvolic acid	Cephalosporin P_1	Fucidin	EPF	Tetracycl (control)
I	21	22	26	22	31	0	0	13	(+)	33	(+)	0	0	0	30
II	20	23	26	23	13	0	0	0	0	15	0	0	0	0	15
III	0	22	25	22	9	0	12	12	0	30	0	0	0	0	30
IV	21	2	25	20	28	0	0	0	0	29	0	0	0	0	27 5
V	22	21 5	25	[illegible]	29	0	0	0	0	30	0	0	0	0	28
VI	22	25	5	23	27 5	0	0	0	0	30	0	0	0	0	28

against pneumococci and β hemolytic streptococci (but not against α hemolytic streptococci) judging from the antibacterial spectra EPF is not identical with helvolic acid Chemical studies are in progress to ascertain whether EPF is identical with any of the other two antibiotics

Several derivatives of helvolic acid cephalosporin P_1 and fusidic acid with antibiotic activity have been produced in laboratories some of these derivatives have also been found as naturally occuring antibiotics produced by fungi With one exception (hydrogenation of the isolated double bond in the side chain of fusidic acid and cephalosporin P_1) all modifications of the molecular structure of these antibiotics hitherto reported have resulted in compounds with lower activity against the test bacteria (*Burton et al* 1955) *Godtfredsen et al* 1965) *Okuda et al* 1966) *Janssen & Vanderhaeghe* 1967)

Beside the above mentioned antibiotics and their derivatives other compounds exist which have a steroid skeleton and possess antibiotic properties polyporenic acid A and C eburicoic acid and viridin The two first mentioned substances have antibacterial activity but their antibacterial spectra are different from that of EPF polyporenic acid A is about 50 times more active against staphylococci than against *Clostridium* spp (*Efimenko et al* 1961) whereas EPF is about equally active against both (*Wallerstrom* 1967) Eburicoic acid and viridin are antifungal antibiotics (*Harvey et al* 1967) — It is noteworthy in this connection that even some animal steroids possess antimicrobial properties deoxycorticosterone for instance in a concentration of 80–250 mcg/ml is reported to inhibit the growth of dermatophytes yeasts and moulds as well as of gram positive bacteria gram negative bacteria with the exception of *Neisseria catarrhalis* are much less susceptible (*Lester & Hechter* 1958) *Maxwell et al* 1960) *Chattaway & Townsley* 1962)

The patterns of antibiotic activity of helvolic acid cephalosporin P_1 and fucidin found in this study were in agreement with literature data on these antibiotics (2 4 5 9 10 11 18 21) The activity of helvolic acid compared with that of cephalosporin P_1 was lower than what was reported by *Burton & Abraham* (1951) who found that cephalosporin P_1 was nearly twice as active against staphylococci as helvolic acid *Ritchie et al* (1951) on the other hand found cephalosporin P_1 to be 4 to 8 times more active than helvolic acid against the same bacterial species The results in the present study are closer to the latter value A possible explanation is that the sample of helvolic acid used in this study contained a small amount of 7 deacetyl helvolic acid as an impurity This substance is produced by fungi but may also be obtained on partial hydrolysis of helvolic acid it is also an antibiotic (synonym helvolinic acid) though its activity is only about one eight of that of helvolic acid (*Burton et al* 1956) According to *Okuda et al* (1966) its antibacterial spectrum is similar to that of helvolic acid

The question whether EPF is mainly bacteriostatic or mainly bac

tericidal was studied in two different ways When the antibiotic was allowed to act on a small amount of staphylococci for 24 h the minimal inhibitory concentration was 0 5 U/ml but the minimal bactericidal concentration was considerably higher 15 U/ml (test technique I) However repeated viable counts in broth cultures of staphylococci during the first 24 h of exposure (test technique II) revealed in most instances a slow decrease in the number of organisms even at a con centration of 5 U/ml – As to steroid antibiotics *Godtfredsen et al* (1962 a) who used the latter technique and small inocula found that 10 mcg/ml of fusidic acid had a bactericidal effect on staphylococci *Garrod & Waterworth* (1956) who used the former technique found the same concentration of cephalosporin P_1 to have bactericidal ac tivity when the inoculum used was small (5 × 10^5 staphylococci per ml or less) when larger inocula were used surviving organisms were found *Chain et al* (1943) using heavy inocula found the m i c of helvolic acid on staphylococci to be 1 5 mcg/ml but the m b c was more than 25 mcg/ml

EPF resistant mutants of staphylococci were readily produced by culturing the strains on media containing EPF Mutants with a high degree of resistance were produced also on media with a very low con centration of EPF and in experiments with gradient plates colonies growing on different gradient levels of the plate showed zones of com parable sizes when tested against EPF in the agar diffusion test De velopment of resistance of this antibiotic is thus of the streptomycin or one step type rather than of the step wise (penicillin) type De velopment of resistance to fucidin is also claimed to be a one step mutation

The development of resistance to EPF by staphylococci did not change their susceptibility to a number of other antibiotics The find ing of resistance to tetracycline in some EPF resistant staphylococcal strains in an earlier investigation (*Wallerstrom* 1967) was thus in cidental – The conclusion by *Godtfredsen et al* (1962 b c) that fuci din resistant strains lose their ability to coagulate human plasma could not be verified in this study If it had been it would have fitted in with the observation that all EPF resistant staphylococcal strains found in skin lesions due to *Epidermophyton floccosum* were coagulase nega tive (*Wallerstrom* 1968) this has to be explained in some other way

EPF resembles helvolic acid cephalosporin P_1 and fucidin also in some other respects its antibacterial activity is reduced in the pre sence of serum and increased by acidification of the test media (*Wal lerstrom* 1969) Inhibition by serum and increased activity at lower pH is reported for all of the three steroid antibiotics mentioned

SUMMARY

The antibacterial spectrum of the Epidermophyton factor (EPF) which is produced by the skin pathogenic fungus *Epidermophyton floccosum* was compared with those of three steroid antibiotics helvolic acid cephalosporin P_1 and fucidin The spectra of the last two antibiotics were very similar whereas helvolic acid was relatively more active than EPF against *inter alia* β streptococci and pneumococci Staphylococci and corynebacteria readily developed resistance to EPF *in vitro* Strains of *Staphylococcus aureus* cultured on media containing EPF or fucidin were found to be resistant to each of these substances as well as to helvolic acid and cephalosporin P_1 In moderate concentrations EPF had bactericidal effect on staphylococci The frequency of resistant mutants in a staphylococcal population was 1 out of 10^7–10^9

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tericidal was studied in two different ways When the antibiotic was allowed to act on a small amount of staphylococci for 24 h the minimal inhibitory concentration was 0 5 U/ml but the minimal bactericidal concentration was considerably higher 15 U/ml (test technique I) However repeated viable counts in broth cultures of staphylococci during the first 24 h of exposure (test technique II) revealed in most instances a slow decrease in the number of organisms even at a con centration of 5 U/ml – As to steroid antibiotics *Godtfredsen et al* (1962 a) who used the latter technique and small inocula found that 10 mcg/ml of fusidic acid had a bactericidal effect on staphylococci *Garrod & Waterworth* (1956) who used the former technique found the same concentration of cephalosporin P_1 to have bactericidal ac tivity when the inoculum used was small (5×10^5 staphylococci per ml or less) when larger inocula were used surviving organisms were found *Chain et al* (1943) using heavy inocula found the m i c of helvolic acid on staphylococci to be 1 5 mcg/ml but the m b c was more than 25 mcg/ml

EPF resistant mutants of staphylococci were readily produced by culturing the strains on media containing EPF Mutants with a high degree of resistance were produced also on media with a very low con centration of EPF and in experiments with gradient plates colonies growing on different gradient levels of the plate showed zones of com parable sizes when tested against EPF in the agar diffusion test De velopment of resistance of this antibiotic is thus of the streptomycin or one step type rather than of the step wise (penicillin) type De velopment of resistance to fucidin is also claimed to be a one step mutation

The development of resistance to EPF by staphylococci did not change their susceptibility to a number of other antibiotics The find ing of resistance to tetracycline in some EPF resistant staphylococcal strains in an earlier investigation (*Wallerstrom* 1967) was thus in cidental – The conclusion by *Godtfredsen et al* (1962 b c) that fuci din resistant strains lose their ability to coagulate human plasma could not be verified in this study If it had been it would have fitted in with the observation that all EPF resistant staphylococcal strains found in skin lesions due to *Epidermophyton floccosum* were coagulase nega tive (*Wallerstrom* 1968) this has to be explained in some other way

EPF resembles helvolic acid cephalosporin P_1 and fucidin also in some other respects its antibacterial activity is reduced in the pre sence of serum and increased by acidification of the test media (*Wal lerstrom* 1969) Inhibition by serum and increased activity at lower pH is reported for all of the three steroid antibiotics mentioned

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INFLUENCE OF INHIBITORS OF DNA AND PROTEIN SYNTHESIS ON THE KINETICS OF DNA UPTAKE IN *NEISSERIA MENINGITIDIS* TRANSFORMATION

By

KAARE JYSSUM

Received 1 v 69

Kinetic measurements of the DNA uptake have been performed in most transformable species In *Streptococcus pneumoniae* linear rates for entry terminated by deoxyribonuclease have been found with both single and linked markers (15) There is a little lag before the first detected entry of each marker The addition of DNA to competent *B subtilis* cells is followed by an unmeasurably rapid irreversible attachement of DNA to the cells (8 18) Subsequently a period of approximately 1 minute ensues during which the potential transformants are sensitive to deoxyribonuclease The lag period which is independent of the single marker selected is considered to represent the time necessary for entry of a length of DNA which is long enough to participate in a recombination event The process of uptake of DNA in *Haemophilus influenzae* cells seems to be much more rapid than in pneumococcus and in *B subtilis* (30) But also in this microbe there seems to be a linear increase of transformants with time The uptake curve does not go through the origin but extrapolates to a value of about 2 5 sec when saturating concentrations of DNA have been used

In *Neisseria meningitidis* there seems to be a slower rate of DNA uptake in the cell than in the other systems more thoroughly examined and no lag has been observed before the first detected entry of a marker (5 20) The purpose of this investigation has been to study these differences in kinetics More specifically the following questions have been posed What is the metabolic situation during DNA uptake in competent cells of this species and what are the metabolic requirements for such uptake?

MATERIALS AND METHODS

Strains

The following mutants were obtained from the wild type strain MI of sero group B as previously described (14) MI 18 *his gly cp* MI 8 *his arg cp* MI 5 *his hom cp*

Men el A E Wintersteiner O & Hoogerheide J C The isolation of gliotoxin and fumigacin from culture filtrates of *Aspergillus fumigatus* J Biol Chem *152* 419–429 1944

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Stewart G T Steroid antibiotics Pharmakotherapia (Munich) 2 137–148 1964

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Wallerström A Production of antibiotics by *Epidermophyton floccosum* III Course of formation and some properties of the Epidermophyton factor (EPF) *ibid* 75 435–446 1969

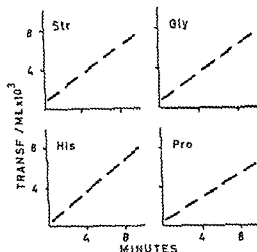


Fig 1

Linear accumulation of singly marked transformants in stirred mixtures of bacteria and DNA at 37° Calculated regression line for Str r is $x = 1.44y - 1.01$ for Gly $x = 1.46y - 1.00$ for His $x = 1.78y - 0.26$ for Pro $x = 1.73y - 1.03$

becomes likely that they extrapolate to a point before zero time i e to a time before the transforming DNA has actually been added From statistical analysis of covariation there appeared no objection against the assumption that the calculated straight lines represent the experimental values

Interference with the Linearity of the Uptake Curve

The kinetic curves of Fig 1 demonstrate the pattern observed when the recipient cells are in the early logarithmic phase of growth when DNA is added Manipulations which reduce the metabolic activity of the cells had a pronounced influence on the form of the curve When the metabolic activity was temporarily reduced by a cold shock before the addition of DNA a pronounced lag was obtained (Fig 2) during which a gradually increasing rate of appearance of transformants occurred

2 4 dinitrophenol is a potent inhibitor of oxidative phosphorylation with activity also on the metabolism of *N meningitidis* (13) Preincubation with 2 4 dinitrophenol had two effects on the transformation curve Fig 3 shows that a significant lag occurred before the appearance of the first transformants Furthermore the rate of appearance of transformants was reduced Further experiments showed that these effects were independent of any particular genetic trait among those tested but seemed to be characteristic of the *N meningitidis* transformation process in general when carried out under these conditions

Ml 48 *his cys cp* Ml 6 *his pro cp* Genetic competence is indicated by the symbol *cp* and incompetence which does not revert to competence by *cp* (1?) The *str* marker was a single step high level resistant mutant (11)

Media

Blood agar or heart infusion broth (HIB Difco) agar was used as solid complete medium Fluid complete medium was brain heart infusion broth (BH Difco) The basal media were those previously used (11)

Genetic Procedures

DNA preparation and transformation procedures followed the previously described technique (11) In the time course experiments the technique was slightly modified Phenotypically competent cells were obtained by inoculating the recipient strain in BH broth from an overnight culture on blood agar and following the absorbance until early logarithmic growth (11) The cells were harvested by centrifuging for 20 minutes at 4000 rpm and resuspended in saline to contain approximately 10^8 col forming units per ml 10 ml HIB with 0 005 M CaCl were inoculated with 1 ml of the suspension and incubated at 37 in a shaker for 20 minutes whereupon the appropriate inhibitor was added usually in a volume of 0 1 ml After 5 minutes 0 5 ml DNA dilution was added usually containing 50 μg/ml in NaCl citrate buffer Samples were removed at suitable intervals to tubes containing deoxyribonuclease (DNase) and $MgSO_4$ such that the final concentration of the enzyme was 50 μg/ml The DNase treated samples were assayed for transformants as previously described (11 14) The number of transformed units was calculated from counts on three plates made in parallel Suitable dilutions of the cultures were plated for the calculation of colony forming units Appropriate controls were included for revertants and for the detection of contamination Additional technical procedures or modifications have been described in the experimental section

Analysis of Covariation

In this paper the number of transformants obtained have been related to the time of DNA exposure This analysis of covariation followed the statistical principles previously used (10) When the graphs indicated that straight lines had been obtained the equations of the hypothetical lines which be t fitted the experimental results were determined by the method of least squares The coefficient of correlation (r) was calculated and discussed as to reality The standard error was calculated but since the actual ob ervation series usually consisted of less than 30 observations it had to be discussed according to the distribution of t The value $t = r/e_r$ was compared with a table showing the distribution of t If this preliminary analysis evinced no objection against the assumption that the experimental results represented the computed line the conformity of the experimental data with the calculated line was usually tested by the analysis of variance In order to do thi values to points in the regression line were calculated from the equation found (10)

RESULTS

Linear Accumulation of Singly Marked Transformants

Experiments were designed to study the appearance of *N meningitidis* transformants as a function of the duration of the contact of recipient cells and DNA Transformation of the mutants with Str r prototrophic DNA was first carried out at 37 Deoxyribonuclease was used to terminate the transformation It was observed that all types tested of singly marked transformants accumulate approximately linearly with duration of exposure to DNA as already shown for streptomycin restistance (5 20) When the curves are studied (Fig 1) it

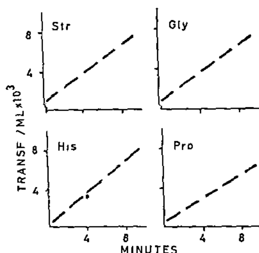


Fig 1

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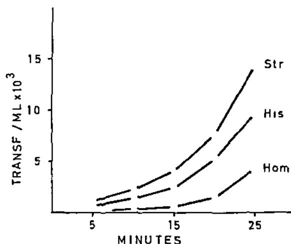


Fig 2

Interference with the linearity of the uptake curve by cold shock The recipient cells were cooled in ice water for 10 minutes and transferred to 37 DNA was added to the cells after temperature equilibration

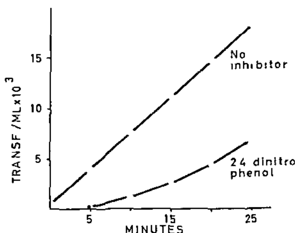


Fig 3

Interference with the kinetics of appearance of transformants to Str r by 1×10^{-3} M 2 4 dinitrophenol added 5 minutes before tran forming DNA

Establishment of a Lag Period Characterizing the Transformation Curve

To obtain further orientation concerning the more exact metabolic requirements for DNA uptake and transformation inhibitors of DNA replication and protein synthesis were tested

Hydroxyurea which is bacteriostatic in *E coli* seems to inhibit DNA synthesis in concentrations which do not affect RNA synthesis or pro tein metabolism (25 26) When competent cultures of *N meningitidis* in the early logarithmic growth phase are pretreated with hydroxyurea for 5 minutes before the addition of transforming DNA a significant

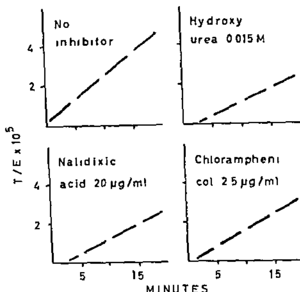


Fig 4

Reduction in the rate of appearance of transformants to Str r in the presence of hydroxyurea nalidixic acid or chloramphenicol The inhibitors were added 5 minutes before the transforming DNA

effect is observed in the kinetic curves (Fig 4) Furthermore it seems that all concentrations have the same approximate effect provided that they are not too high to permit a sufficient dilution of the chemical during the plating for essay of transformants This effect is a reduction in the rate of appearance of transformants to around one half of that found in the control system without hydroxyurea Calculations indicate that the experimental data obained in the presence of hydroxyurea may still be described by a linear regression line but when this line is extrapolated to zero transformants is is found that the point of intersection is at about 2 minutes Thus both the slope of the curve and the point of intersection has been changed This change in the kinetics seems to be common to all markers tested (Fig 5)

Nalidixic acid a potent inhibitor of DNA synthesis has a killing action on *E coli* But it has been shown that the inhibition can be reversed by transfer to a drug free medium provided that the transfer takes place within a fairly short period such as 60 minutes in *E coli* (6) From Fig 4 is seen that nalidixic acid added to competent *N meningitidis* cultures reduces the rate of appearance of transformants to approximately half of that in the control system Also in the presence of this inhibitor the point of intersection is moved to the right to approximately 2 minutes When nalidixic acid is added to the system it is particularly important to use concentrations which are sufficiently diluted out during the essay of transformants It is es

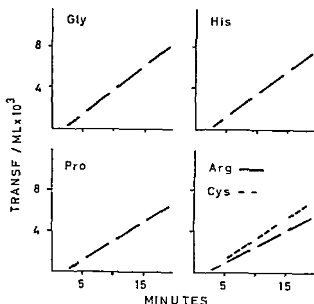


Fig 5

Lag period in the appearance of the first transformed marker in stirred mixtures of bacteria and transforming DNA in the presence of 0 015 M hydroxyurea Calculated regression line for Gly $x = 2\ 14y + 2\ 07$ for His $x = 2\ 56y + 1\ 82$ for Pro $x = 2\ 98y + 1\ 92$ for Arg $x = 3\ 16y + 2\ 38$ for Cys $x = 2\ 54y + 1\ 68$

sential to use short times of exposure to the drug Even the concentration 20 µg/ml results in some killing particularly when the cells have been exposed to nalidixic acid for more than 10 minutes Therefore it becomes necessary to calculate the effiency of transformation T E in these experiments

Chloramphenicol was added in varying concentrations to competent cells of *N meningitidis* in the early logarithmic phase 5 minutes before the addition of DNA Fig 4 shows that also chloramphenicol has a significant effect on transformation with a reduction in the slope of the kinetics curve Even with chloramphenicol the point of intersection seems to be moved a little to the right resulting in a lag before the appearance of the first transformed marker

Since chloramphenicol and hydroxyurea seemed to have the same type of effect on the kinetics the combined effect was compared to that of each of the two drugs It seems that the combined effect is nearly the same as that of hydroxyurea alone while chloramphenicol alone is somewhat less effective (Fig 6)

Effect of the DNA Concentration on the Uptake Curve

The effect of DNA concentration on the kinetic curves obtained in the presence of inhibitors was examined From Fig 7 is seen that in the presence of hydroxyurea insignificant fluctuation occures in the

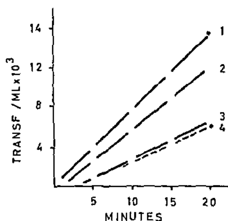


Fig 6

Combined effect of hydroxyurea and chloramphenicol on the rate of appearance of transformants to Str r Curve 1 Control system with no inhibitor Curve 2 2 μg chloramphenicol/ml Curve 3 0 015 M hydroxyurea Curve 4 ? μg chloramphenicol/ml plus 0 015 M hydroxyurea The inhibitors were added 5 minutes before the transforming DNA

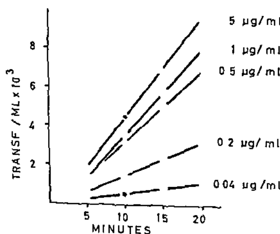


Fig 7

Formation of transformants to Str r in stirred mixtures of bacteria and transforming DNA at indicated concentrations The system was preincubated with 0 015 M hydroxyurea for 5 minutes before the addition of transforming DNA

lag period over a range of DNA concentration over 1000 An increasing rate of uptake was observed up to about 2 μg DNA per ml above which there is little further increase (Fig 8) Optimal concentrations of DNA are nearly the same as those obtained in *N meningitidis* without the addition of inhibitors (20) These results are not very different from those obtained in *H influenzae* (30)

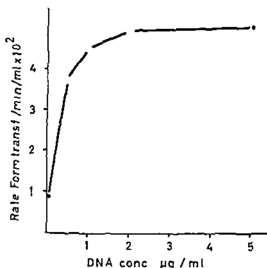


Fig 8

Relationship between rate of formation of transformants to Str r and concentration of transforming DNA in the presence of 0 015 M hydroxyurea

DISCUSSION

There are several arguments for a metabolically primed active transport mechanism for DNA based on experiments with *Haemophilus* (2 30) and *B subtilis* (32) An energy source such as glucose is required and inhibitors of oxidative phosphorylation such as 2 4 dinitrophenol or cyanide prevent irreversible uptake of DNA Also in *N meningitidis* an energy source is required for the uptake of transforming DNA (20 K *Jyssum* Unpubl results)

The effects of 2 4 dinitrophenol on transformation in *N meningitidis* are similar to those observed in *H influenzae* (29 30) Stuy and Stern (30) assumed that the lag period and the decreased rate of DNA uptake are both parameters for the uptake process and suggested that the chemical interferes with the flow of energy required for the DNA penetration step

But if energy production is necessary we need information regarding the particular functions for which the energy is required To obtain such information inhibitors of protein and DNA synthesis were tested During these experiments it has been assumed that the effects on the particular biosyntheses are the same in *N meningitidis* as those found in *E coli* (21 25 26 6)

There is a great deal of evidence indicating that DNA synthesis is not necessary for the uptake of DNA in *B subtilis* (22) or in pneumococcus (7) The present experiments with hydroxyurea and nalidixic acid may be taken to indicate that also in *N meningitidis* no DNA synthesis is actually required

Preincubation of competent cells of *B subtilis* with high concentra

tions of chloramphenicol affects no aspect of the uptake kinetics (18) The effects of chloramphenicol reported above show that this drug cannot block the uptake of DNA during the *N meningitidis* transformation either implying that no protein synthesis is necessary

But even if nalidixic acid hydroxyurea and chloramphenicol do not stop transformation in *N meningitidis* they all bring about a significant reduction in the rate of appearance of transformants It may be assumed that this effect is primarily concerned with the metabolic situation which exists during the process of transformation

There are many indications that the competent state occurs when the cells have reached a resting state in most systems examined In *Haemophilus* as well as in *B licheniformis* competence seems to be induced by a down shift (17 29) Such procedures in meningococci only decrease the transformation frequencies (20) *Nester* (23) demonstrated that competent cells of *B subtilis* both before and after uptake of DNA are resistant to killing by penicillin for several hours implying that competent cells are neither multiplying nor synthesizing cell wall material Such differences have not been detected between competent and incompetent cells of *N meningitidis* (19)

Maximum competence in pneumococcus *H influenzae* and *B subtilis* is found in the latter part of the growth cycle and the beginning of the stationary phase (1 27 29) In contrast *N meningitidis* shows no sharp or high wave of competence The cells are transformed all through the growth cycle (5) but with maximum transformation efficiency in the early logarithmic phase (20)

In pneumococci there is a period of 1–2 hours between expression and onset of division of transformants (9) Newly transformed cells of *B subtilis* exhibit a lag in multiplication and expression of newly transformed markers of 3–4 hours (24 28) which cannot be attributed to lack of integration as donor DNA seems to become integrated within 30 to 45 minutes after it has been added (3 24 31) In meningococci there is virtually no lag between expression and onset of division (5 20)

Thus there are several indications which point to a high metabolic activity of the competent cells of meningococci The observed effects of hydroxyurea nalidixic acid and chloramphenicol could therefore mean that DNA replication protein synthesis as well as cell division actually take place during the process of transformation in this species although these functions are not required

The effect of chloramphenicol could indicate that enzymes which are active in the transformation are present at the time of DNA addition to competent cells but that they increase in quantity after the addition It could also mean that a new uptake process is induced in addition (16) Such a system does not seem to exist in *B subtilis* (18) On the other hand chloramphenicol does not seem to add to the inhibiting effect of hydroxyurea This may of course mean that the

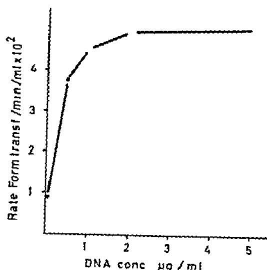


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Preincubation of competent cells of *B subtilis* with high concentra

interpreted to mean that in competent cells of this microbe DNA replication and cell division continue during the process of transformation although these functions are not actually required for the uptake of transforming DNA

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latter agent also inhibits protein synthesis But if this is not the case one would expect that the common target is DNA synthesis Thus while hydroxyurea and nalidixic acid interfere with the DNA synthesis itself cloramphenicol may block the initiation of a new round of replication (21) There is also evidence that chloramphenicol has this effect in *N meningitidis* (*K Jyssum, J Bact 99* 757–763 1969) The difference between the chloramphenicol assay and the combined assay as well as the hydroxyurea experiment may then represent the completion of chromosome replication which had already been initiated before the addition of chloramphenicol

A fundamental difference between the transformation kinetics of *N meningitidis* without the addition of metabolic inhibitors and those exhibited by pneumococci (15) *B subtilis* (18) and *Haemophilus* (30) is the apparent lack of a lag period of entry of the first detectable marker when the transformation is terminated by deoxyribonuclease This pattern seems to be characteristic for *N meningitidis* (5 20) and other transformable *Neisseria* species as well as for *Moraxella* species (4)

It is easy to understand why a replication and a cell division during the time of observation may increase the slope of the transformation curve but it becomes necessary to explain why the lag period is obscured In the technique used nearly the same time lapsed between the addition of DNA and the final dilution and plating for all samples Since the cells are actively growing the chance of replication of a marker will be very high if it inters the cell early in the observation period and low if it enters late We may for instance assume that the hypothetical transformation curve $x = y + 1$ is obtained in the presence of inhibitor with x as the time before addition of deoxyribonuclease and y as the number of transformants obtained If one replication may take place of markers that have entered at the time $x = 2$ and the chance of replication is reduced to ½ for each successive time unit the following curve is obtained when the inhibitor is removed $x = \frac{3}{4} y + \frac{1}{2}$ Thus the slope as well as the point of intersection is changed

SUMMARY

The kinetics of appearance of transformants as a function of time of exposure to DNA has been studied in *Neisseria meningitidis* The addition of hydroxyurea or nalidixic acid as well as of chloramphenicol reduces the rate of formation of transformed cells A lag period appears before the first detected entry of each marker The length of this lag is approximately 2 minutes in the presence of hydroxyurea or nalidixic acid and seems to be independent of the genetic trait transformed The observed changes in kinetics have been discussed in relation to p[illegible]ous findings [illegible]ncerning the development of competence and the e[illegible]ssion of trans[illegible]rmed traits in *N meningitidis* The data have been

interpreted to mean that in competent cells of this microbe DNA replication and cell division continue during the process of transformation although these functions are not actually required for the uptake of transforming DNA

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PRESENCE AND PERSISTENCE OF AUSTRALIAN ANTIGEN IN A SWEDISH HEPATITIS SERIES

By

E. NORDENFELT and L. KJELLÉN

Received 21. v. 69

During a search for isoprecipitins in human sera *Blumberg et al.* (1965) incidentally found a new serum protein. The isoantigen has been called Australian antigen because it was first found in serum from an Australian aboriginal. Later investigations (*Alter et al.* 1966, *Blumberg et al.* 1967) have shown that it is different from the lipoprotein isoantigen systems. Australian antigen (Au) is rare (about 0.1 per cent) in normal American populations. It often occurs transiently in patients with acute viral hepatitis (in 13 per cent with infectious hepatitis and in 34 per cent with post transfusion hepatitis). Au antigen also occurs in patients with some forms of leukaemia and Down's syndrome. *Bayer et al.* (1968) recently reported Au antigen in association with pleomorphic particles (about 200 Å) with a structure compatible with that of a virus.

Prince (1968) has demonstrated an antigen termed SH that reacted in the immunodiffusion test with serum from multiply transfused patients. The SH antigen was detected in blood during the incubation period before the onset of chemical or clinical signs of hepatitis. Preliminary results suggested that the SH antigen is located on a virus-like particle with an electron microscopic appearance similar to that of arbovirus and with a diameter of approximately 25 mμ. SH antigen has been described as similar to Au antigen (*Prince* 1968).

Okochi & Murakami (1968) recently reported the occurrence of Au antigen in Japanese. Like *Blumberg et al.* and *Prince* they found Au antigen most often in patients with hepatitis (in 15.2 per cent with infectious hepatitis, 12.9 per cent with post transfusion hepatitis). The frequency in blood donors in Tokyo was estimated at 1 per cent.

The present study reports the frequency of Australian antigen in three selected hepatitis series in Sweden. Blood specimens have been tested from cases treated at the clinic for Infectious diseases in Lund

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Prince (1968) has demonstrated an antigen termed SH that reacted in the immunodiffusion test with serum from multiply transfused patients The SH antigen was detected in blood during the incubation period before the onset of chemical or clinical signs of hepatitis Preliminary results suggested that the SH antigen is located on a virus like particle with an electron microscopic appearance similar to that of arbovirus and with a diameter of approximately 25 mμ SH antigen has been described as similar to Au antigen (*Prince* 1968)

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The present work was supported by the Swedish Medical Research Council (project B 70-16X-2865-01)

TABLE 1

The Appearance of Au Antigen before Signs of Clinical Hepatitis

Patient	Date	7/10	22/10	31/10	5/11	19/11	27/11	4/12	11/12
I E A	Au	—	—	—	+	+	+	+	+
	GPT	14	17	15	14	1[illegible]	40	*310*	*3[illegible]*
	Date	4 12	11/12	18/12	27/12	3/1	14/1	[illegible]/1	1[illegible]/2
B A	Au	—	—	—	+	+	+	+	+
	GPT	38	12	2[illegible]	19	20	14	*77*	[illegible]
	Date	18/12	27/12	3/1	14/1	[illegible]/1	1[illegible]/2	3/3	
4 S	Au	—	—	—	+	+	+	+	
	GPT	13	15	1[illegible]	9	10	9[illegible]	*5[illegible]*	

Abnormal GPT values are in italics

ber 1968 but when last tested two months later neither had clinical signs of hepatitis nor increased GPT been observed

It should be mentioned that in all the 14 patients who were receiving treatment with dialysis and in whom the reaction was or turned positive the reaction remained so throughout the investigation period Several have now exhibited Au antigen for at least 4 months

The 9 Au negative patients showed no signs of hepatitis during the investigation period One of them had hepatitis 2 months before the start of this investigation

The 4 cases of hepatitis among the staff who have been studied are included in the material from the clinic for infectious diseases Two of them have been Au positive

TABLE 2

Presence of Au Antigen in Patients Treated for Hepatitis on the Clinic for Infectious Diseases

		Cases	Au	Au
Clinical diagnose	Infectious hepatitis	8	2	6
	Serum hepatitis	13	8	5
		21	10	11

Au Antigen among Patients Treated at the Clinic for Infectious Diseases

Blood samples from most patients treated for hepatitis in the clinic for Infectious diseases have been tested for Au antigen since the summer 1968 Until now we have studied 40 sera from 21 patients The results are given in Table 2

The blood samples in this group were obtained in different stages of the disease In none of the ten Au positive cases were blood samples available from the early "incubation time From 7 of them however

the Medical clinic B (dialysis department) in Lund and the Juvenile Rehabilitation school at Raby (addicts)

MATERIAL AND METHODS

Clinical specimens Since October 1968 blood samples have been taken once a week from the 23 patients treated in the dialysis department

The blood specimens from patients treated for hepatitis at the unit for infectious diseases were taken in acute phase and/or in convalescens

Of 42 pupils at Råby in October 1968 28 were addicts In a clinical study of the occurrence of hepatitis among the pupils blood samples were taken at regular intervals

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Method Double immunodiffusion in agarose according to *Ouchterlony* (1958) Agarose 0 9 per cent was used according to *Prince* (1968)

Glutamic pyruvic transaminase (GPT) The GPT activity in serum was deter mined fluormetrically in an autoanalyzer at the laboratory of Clinical Chemistry University Hospital Lund Values above 40 units are regarded as abnormal

Bilirubin Bilirubin in serum was determined in an autoanalyzer at the laboratory of Clinical Chemistry University Hospital Lund Values above 1 2 mg/100 ml are regarded as abnormal

Terminology Most of this work is done with reagents supplied by Dr Prince but we have for practical reasons preferred to refer to the antigen as Au antigen in accordance with the terminology of Blumberg et al In our hands reactions of identity were obtained when the reagents from the two laboratories were cross tested

RESULTS

Au Antigen among Patients Treated at the Dialysis Department

In the summer and fall of 1968 verified hepatitis occurred in patients or members of the staff Since then the patients and the personal have been regularly examined for increased GPT and presence of Au antigen The clinical symptoms of hepatitis were usually mild The diagnoses have been based on increased GPT

From Oct 1968–Jan 1969 105 sera from 23 patients receiving treat ment with dialysis were examined for Au antigen Blood samples from 14 of these patients were found to contain Au antigen Judging from the GPT and other findings all except 1 of these 14 had or had shortly before had hepatitis

The Au positive cases can be divided into two groups one with Au antigen continously from the beginning and one where the test for Au turned positive during the investigation

The first group comprised 8 patients including 5 with clinical signs of hepatitis and increased GPT during the period of the investigation The other 3 had hepatitis with increased GPT 1–2 months before the investigation period

The second group consisted of 6 patients In three of these six did the Au antigen appear before signs of clinical hepatitis The results of the tests on these three patients are given in Table 1

In two of the six cases appearance of Au antigen was accompanied by an increase of the GPT The sixth case became Au positive in Decem

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B A	Au	—	—	—	+	+	+	+	+
	GPT	38	12	22	19	20	14	77	55
	Date	18/12	27/12	3/1	14/1	29/1	12/2	3/3	
A S	Au	—	—	—	+	+	+	+	
	GPT	13	15	12	9	10	*95*	*56*	

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In two cases it has thus been possible to detect a transient occurrence of Au antigen In the other two the antigen was demonstrated in the first available blood sample in one (J V S) of them before the appearance of clinical and biochemical signs of hepatitis In all 4 cases the antigen disappeared while signs of hepatitis still persisted

DISCUSSION

Au antigen is very rare in normal populations in USA as well as in northern Europe (*Blumberg et al* 1968) Owing to shortage of antisera no attempts have been made to assess the frequency of Au antigen in Swedish populations

Our results confirm the earlier reports on Au antigen (*Blumberg et al* 1968 *Prince* 1968 *Okochi & Murakami* 1968) and its close association with serum hepatitis Of the 34 patients with serum hepatitis in our material 25 have been Au positive The association between Au antigen and serum hepatitis was thus high and practically 100 per cent in the patients receiving treatment with dialysis This is in good agreement with the finding by *London et al* (1968) In agreement with *Prince* (1968) and *Okochi & Murakami* (1968) the appearance of Au antigen has sometimes been demonstrated to precede the onset of clinical signs of hepatitis

In the hepatitis patients without complicating disorders the Au antigen was detected only transiently and disappeared earlier than the clinical signs of hepatitis This is in sharp contrast to the outcome obtained among patients receiving treatment with dialysis Once demonstrated the Au antigen did not disappear and all cases have remained Au positive during the 4–5 months investigation period The persistence of Au antigen may in some way be connected with uremia or the loss of normal renal function Of the 14 Au positive patients 6 have undergone bilateral nephrectomy the others were anuric All had been dialysed twice a week It is interesting to note that *Okochi & Murakami's* (1968) series included one uremia patient—still under observation —who had had the antigen for 5 months

Like *Blumberg et al* (1967) and *Okochi & Murakami* (1968) we have seen some Au positive cases among patients with the diagnosis of infectious hepatitis

The nature of Au antigen is still obscure *Bayer et al* (1968) as well as *Prince* (1968) have reported particles about 200 A size with a structure compatible with that of a virus It might be mentioned that similar particles have been seen by us in an electron microscopical study The nature of these particles is receiving attention

SUMMARY

The occurrence of Au antigen among three different groups of patients has been studied A close association betwe n Au antigen and hepatitis

repeated samples have been tested The antigen was demonstrable for only a short time despite persistance of clinical signs of hepatitis and increased GPT

TABLE 3
Raby School Pupils without Signs of Clinical Hepatitis Tested on One Occasion for Presence of Au Antigen and Antibodies

	No	Au	Au	Antibodies to Au antigen
Addicts	28	1	27	0
Alcoholics	12	0	12	0
Other	2	0	2	0
	42			

TABLE 4
Four Au Positive Addicts Tested Repeatedly During the Course of their Hepatitis for Presence of Au Antigen

Patient	Date	Au	Bilirubin mg/100 ml	GPT units†
KK	6/12	−	0.4	22
	27/12	+	3.3	1480
	10/1	−	2.4	1034
	25/2	−	0.8	30
KH	30/12	+	2.2	825
	9/1	+	8.5	1134
	24/1	−	3.5	517
	4/2	−	1.3	46
JAS	13/12	+	0.4	24
	2/1	+	0.8	550
	10/1	+	1.6	1144
	24/1	+	1.4	462
	5/2	−	0.9	87
TI	29/10	−	1.0	32
	5/2	+	6.5	632
	14/2	+	6.3	400
	6/3	−	1.2	48

Normal limit ≦ 1.2 mg/100 ml
† Normal limit ≦ 40 units

Au Antigen among Pupils at Raby School

Blood samples taken at one occasion from the pupils at the school were screened On that occasion none of the pupils showed overt clinical signs of hepatitis 42 pupils were tested (Table 3)

As seen from the table the reaction for Au was positive in 1 case

In the continued study 8 addicts were repeatedly examined in connection with the appearance of hepatitis Four of these were Au positive The results of the tests are given in Table 4

In two cases it has thus been possible to detect a transient occurrence of Au antigen In the other two the antigen was demonstrated in the first available blood sample in one (I A S) of them before the appearance of clinical and biochemical signs of hepatitis In all 4 cases the antigen disappeared while signs of hepatitis still persisted

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SUMMARY

The occurrence of Au antigen among three different groups of patients has been studied A close association between Au antigen and hepatitis

is confirmed it was demonstrated in about 70 per cent of patients with serum hepatitis A persistence of Au antigen in patients receiving treatment with dialysis but not in others is found

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SEROLOGICAL PROPERTIES OF AQUEOUS ETHER EXTRACTED ENDOTOXIN FROM *NEISSERIA GONORRHOEAE*

By

J A MÆLAND

Received 24 iv 69

Previous studies of various endotoxin preparations from a *N gonorrhoeae* strain using indirect haemagglutination techniques revealed two antigenic determinants (10) One of these determinants was of carbohydrate nature the other of protein nature They were designated determinant *a* and determinant *b* respectively Results obtained with the aqueous ether extract indicated that both determinants belong to the endotoxin complex (11) The aqueous ether endotoxin contained lipid sugar and protein the latter being the dominating component (12) Glucose galactose glucosamine and heptose were detected (12)

The present study of the aqueous ether endotoxin is concerned with the erythrocyte sensitizing activity the nature of the antibodies to the endotoxin and their reactivity in various tests

MATERIALS AND METHODS

N gonorrhoeae strain Strain 8551/64 was employed Cultivation and harvesting were performed as described previously (8)

Extraction method The bacteria were extracted with aqueous ether and the endotoxin was purified according to the procedures described previously (10 11)

Antisera Rabbit antisera to whole gonococci (anti Gc) were prepared as described earlier (8)

Antiserum to rabbit erythrocytes sensitized with alkali treated endotoxin (anti SRE) was prepared as follows The endotoxin was treated with 0 005 N NaOH at 37 C for 18 hrs neutralized and dialysed against 1/150 M phosphate buffered saline pH 7 2 Equal volumes of a solution containing 0 5 mg per ml of endotoxin and a one per cent suspension of erythrocytes from the animal to be immunized were mixed and kept at 37 C for 30 mins The erythrocytes were washed 5 times and prepared as a 20 per cent suspension in saline One ml was injected intravenously twice a week for 2 weeks and a last injection was given 4 weeks later

The animals were bled [illegible] after the last injection

Absorption of antisera Antibodies to both the determinants *a* and *b* were removed by absorption of undiluted antiserum with aqueous ether endotoxin Antisera containing antibodies to determinant *a* (anti Gc-*a* anti SRE *a*) but not to determinant *b* were prepared by absorption of the undiluted sera with gonococci treated with periodate (11) Antisera containing antibodies to determinant *b* (anti Gc *b* anti SRE *b*) but not to *a* were prepared by absorption with phenol water endotoxin (10 11)

That complete absorption of the antibodies had been secured was checked by

testing a 1 2 dilution of the absorbed sera against appropriately sensitized erythrocytes (see below)

Absorption of anti Gc serum with erythrocytes sensitized with alkali treated endotoxin was performed by mixing one ml of packed sensitized erythrocytes with 1 6 ml serum diluted 1 32 The mixture was kept at 20 C for 30 mins followed by centrifugation at 1000 $\times g$ for 15 mins

Antiserum to rabbit serum A goat antiserum to whole rabbit serum was kindly provided by Dr *E Glück* Bergen The titre was 16 384 with sheep erythrocytes sensitized with sub agglutinating amounts of rabbit antiserum to sheep erythrocytes A 1 200 dilution of the goat antiserum was employed in the anti γ globulin test

Indirect haemagglutination and haemolysis inhibition of haemagglutination The antigen preparations used for sensitization of erythrocytes are described under Experiments and Results The techniques for the sensitization of erythrocytes and the performance of the tests have been described in earlier reports (8 10) In the indirect haemolysis test fresh guinea pig serum diluted 1 15 was employed as complement

Agar precipitation One per cent of agar (Special Noble) was used The antigen wells were filled with a suspension containing about 100 mg of wet gonococci per ml The suspension had been kept at 4 C for at least 3 days in order to obtain distinct lines in the precipitation test The serum wells were filled with undiluted antiserum The plates were incubated at 4 C for up to 10 days

Immunoelectrophoresis The equipment of LKB Produkter AB Stockholm Sweden was employed The saline suspension of gonococci was added to the antigen wells and a constant voltage of 250 V was applied for 90 mins at 20 C using a 0 06 M veronal buffer pH 8 6 Antiserum was then added and the reaction was observed for 2 days

Agglutination of gonococci Agglutination of untreated gonococcal cells and of gonococci heated at 100 C for 60 mins was performed in tubes Twofold serial dilutions of the antisera were prepared in phosphate buffered saline pH 7 2 in 0 2 ml volumes Equal volumes of a suspension of washed bacteria containing approximately 10^9 organisms per ml were added The tubes were incubated at 4 C for 18 hrs and centrifuged at 1000 $\times g$ for 60 secs the agglutination being recorded after resuspension of the sediment A control without antiserum was included

Complement fixation tests Twofold dilutions of the antiserum were prepared in 0 2 ml volumes Then 0 2 ml of appropriately diluted endotoxin and 0 2 ml containing 2 haemolytic units (100 per cent) of complement were added The mixtures were kept at 4 C for 18 hrs and then at 37 C for 10 mins Thereafter 0 4 ml of a one per cent suspension of erythrocytes sensitized with 2 haemolytic units (100 per cent) of rabbit antiserum to sheep erythrocytes was added After incubation at 37 C for 30 mins the degree of haemolysis was recorded and the results given as the reciprocal of the highest dilution with complete inhibition of haemolysis Antigen and serum controls were included Veronal buffer containing optimal amounts of Ca and Mg was used as diluent (4)

Gel filtration Undiluted antiserum 2 5 ml was subjected to gel filtration on columns of Sephadex G 200 according to the method of *Flodin & Killander* (3) The fractions were tested for activity in the indirect haemagglutination test

Reduction by mercaptoethanol Equal volumes of serum fractions from the Sephadex column and 0 2 M 2 Mercaptoethanol were mixed kept at 37 C for 30 mins and tested for activity without dialysis

Alkali and heat treatment of endotoxin Lyophilized endotoxin was dissolved in various concentrations of NaOH ranging from 0 0025 to 0 05 N The concentration of antigen was one mg per ml The solutions were kept at 37 C for 18 hrs neutralized with HCl dialysed against buffered saline and then adjusted to 0 5 mg per ml of antigen The preparations were used for sensitization of erythrocytes as described below

Heating was performed either in a boiling water bath for 60 mins or in the autoclave (120 C) for 60 mins

Digestion with pronase (B Grade Calbiochem) The digestion was carried out at 37 C for 4 hrs in phosphate buffered saline pH 7 2 with an enzyme to substrate ratio of 1 50 The enzyme was inactivated at 100 C for 5 mins

Oxidation with periodate The oxidation was carried out in the dark at 20 C for 20 hrs with 0 5 mg per ml of endotoxin in a solution of 0 01 M sodium metaperiodate

EXPERIMENTS AND RESULTS

Sensitization of Erythrocytes for Indirect Haemagglutination and Haemolysis

Attempts were made to sensitize normal sheep erythrocytes with solutions containing one mg per ml of untreated endotoxin Erythrocytes treated in this manner were not agglutinated by the anti Gc serum and haemolysis was not observed either when complement was added Apparently sensitization had not been achieved

Endotoxin heated at 100 C for 60 mins did not sensitize erythrocytes On the other hand sensitization was obtained with endotoxin heated at 120 C for 60 mins Solutions containing from 250 to 1000 μg per ml of the heated preparation sensitized erythrocytes for agglutination and haemolysis both with anti Gc *a* and with anti Gc *b* sera

Twofold serial dilutions of each sample of endotoxin treated with alkali (0 0025 to 0 05 N NaOH) were prepared and used for sensitization of equal volumes of a one per cent suspension of sheep erythrocytes Anti Gc *a* or anti Gc *b* sera 0 2 ml containing 8 agglutinating units were then added to 0 2 ml of a 0 5 per cent suspension of the sensitized erythrocytes In this way the minimal amount of antigen sensitizing erythrocytes for agglutination and for haemolysis with 8 units of the antisera could be determined

Anti Gc *a* and anti Gc *b* differed in their reactivity with sensitized erythrocytes depending on the concentration of NaOH used for treatment of the endotoxin (Fig 1) All samples of endotoxin treated with alkali sensitized erythrocyts for agglutination and haemolysis with anti Gc *b* The preparations treated with 0 01 or 0 02 N NaOH had the strongest ability to sensitize erythrocytes with determinant *b*

Anti Gc-*a* in contrast to anti Gc *b* did not agglutinate erythrocytes sensitized with endotoxin pretreated with 0 0025 to 0 01 N NaOH (Fig 1) Treatment with 0 01 N NaOH was necessary to induce haemolysis with anti Gc *a* Endotoxin pretreated with 0 02 to 0 05 N NaOH sensitized erythrocytes for agglutination and haeomlysis with anti Gc *a* the various samples showing almost identical sensitizing activity

The ability of the various alkali treated preparations to sensitize erythrocytes for haemolysis with anti Gc *a* or anti Gc *b* was markedly stronger than the ability to sensitize for agglutination especially with anti Gc *b* Results similar to those described were obtained when rabbit erythrocytes were used instead of sheep erythrocytes

Further experiments were performed to test whether antibodies to determinant *a* combined with erythrocytes sensitized with endotoxin which had been treated with 0 0025 to 0 01 N NaOH Erythrocytes sensitized with these preparations were incubated with 8 agglutinating units of the anti Gc *a* serum and thereafter washed three times The goat anti rabbit serum was then added No agglutination was observed indicating that rabbit antibodies had not combined with the sensitized

testing a 1 2 dilution of the absorbed sera again t appropriately sensitized ery throcytes (see below)

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Titres in Various Tests of Unabsorbed and Absorbed Antiserum to the Aqueous Ether Endotoxin

Test	Antigen	Anti SRE	Anti SRE *a*	Anti SRE *b*	X
Indirect haemaggl	Determinant *a*	512	512	<16	<16
Indirect haemolysis	Determinant *a*	4096	4096	<16	<16
Indirect haemaggl	Determinant *b*	1024	<16	1024	<16
Indirect haemolysis	Determinant *b*	16384	<16	8192	<16
Complement fixation	Endotoxin	256	128	128	<16
Bacterial aggl	Live gonococci	256	128	256	256
Bacterial aggl	Boiled gonococci	256	128	128	16

Anti SRE Antiserum to rabbit erythrocytes sensitized with the aqueous ether endotoxin

Anti SRE *a* Anti SRE after absorption of the antibodies to determinant *b*

Anti SRE *b* Anti SRE after absorption of the antibodies to determinant *a*

X Results obtained with the pre immune serum and with anti SRE absorbed with the endotoxin

shown in Table 2 Each antiserum agglutinated live and heat killed gonococci Absorption of anti SRE with endotoxin reduced its ability to agglutinate boiled gonococci to that of the pre immune serum showing that the endotoxin is an agglutinogen in heat treated bacteria On the other hand unabsorbed and absorbed anti SRE and the pre immune serum agglutinated live bacteria to the same titre Sera from 10 non immunized rabbits were examined for agglutination of gonococci When boiled gonococci were used the titres varied from 4 to 16 and when live bacteria were used from 128 to 256

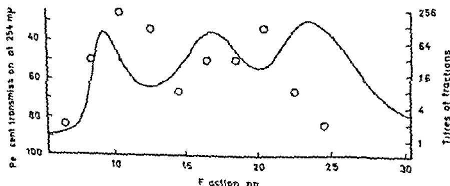


Fig 3

Titre in the indirect haemagglutination test of fractions obtained by gel filtration of rabbit anti SRE serum on Sephadex G 200 column Every two consecutive fractions 5 ml each were combined

—— Per cent transmission at 254 mμ

Titres of the antibodies to determinant *a*

Titres of the antibodies to determinant *b*

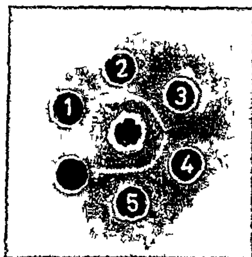


Fig 2
Precipitation pattern formed by gonococci and antisera
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Central well Gonococci

against running tap water and finally against buffered saline Usually 500 μg of the preparation was employed for sensitization of one ml of a one per cent suspension of erythrocytes Anti Gc *b* but not anti Gc *a* agglutinated erythrocytes sensitized in this manner

Either crude or purified endotoxin could be used to produce the sensitizing preparations

Activity in Various Tests of Anti SRE Anti SRE a *and Anti SRE* b, *and Nature of Antibodies to Determinants* a *and* b

In the ring precipitation test a solution containing endotoxin (on mg per ml) formed a precipitate against undiluted anti SRE up to a dilution of 1 32 and up to 1 16 against each of the sera anti SRE *a* and anti SRE-*b* In contrast the endotoxin formed no precipitation line with antisera in the gel diffusion tests (11) However each of the 3 antisera formed one precipitation line with a saline suspension of gonococci (Fig 2) the three precipitation lines fusing into one Anti SRE absorbed with the endotoxin gave no precipitation line with gonococci Using anti SRE or either of the specifically absorbed antisera in the antibody trough immunoelectrophoresis of the bacterial suspension showed a long precipitation arc which extended from the antigen well towards the anode

The results of indirect haemagglutination and haemolysis complement fixation and bacterial agglutination tests are compiled in Table 2 Anti SRE reacted with erythrocytes sensitized with determinant *a* and with determinant *b* The highest titres were obtained in the haemolysis test Anti SRE and each of the specifically absorbed antisera fixed complement in the presence of the endotoxin It was found that at least 12 5 μg of antigen was required to obtain maximal titres in the complement fixation test when all 3 antisera gave the titres

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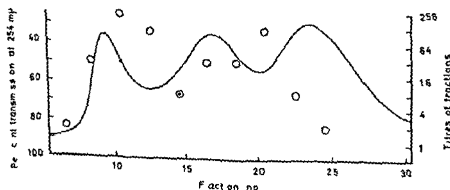


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Titres of the antibodies to determinant *a*
○ Titres of the antibodies to determinant *b*

erythrocytes during incubation with anti Gc-*a* Anti Gc serum was absorbed with erythrocytes sensitized with 0 005 N NaOH treated endotoxin The titre of the antibodies to determinant *b* decreased from 4096 to less than 32 whereas the titre of the antibodies to determinant *a* was 1024 as in the case of the unabsorbed antiserum Accordingly antibodies to determinant *b* combined with the erythrocytes used for absorption while the antibodies to determinant *a* did not

In order to ascertain whether determinant *a* adhered to erythrocytes during incubation with the 0 005 N NaOH treated endotoxin the alkali treated sample was absorbed repeatedly with sheep erythrocytes until the solution no longer sensitized erythrocytes for agglutination or haemolysis with anti Gc serum However the activity of determinants *a* and *b* measured by inhibition of haemagglutination was not significantly reduced by the absorptions The results were thus inconclusive On the other hand this finding suggests that even after treatment with alkali only a number of the endotoxin particles have the capacity to adhere to erythrocytes A rabbit was immunized with its own erythrocytes sensitized with the 0 005 N NaOH treated preparation The antiserum obtained (anti SRE) contained antibodies to both *a* and *b* (see below) Accordingly the erythrocytes had adsorbed both the determinants

TABLE 1

Effect of Treatment with Alkali on the Activity of the Aqueous Ether Endotoxin Determined by Inhibition of Haemagglutination

Normality of NaOH	MID with erythrocytes sensitized with	
	Determinant *a*	Determinant *b*
None	12 5	6 25
0 0025–0 02	12 5	6 25
0 03	12 5	50
0 04 –0 05	12 5	>200

MID Minimal inhibiting dose in μg with 8 agglutinating units of the antiserum

The alkali treated preparations were tested for inhibition of haemagglutination using erythrocytes sensitized with determinant *a* or *b* according to the standard procedures described below Treatment with alkali did not affect the inhibiting activity of determinant *a* (Table 1) The activity of determinant *b* was not changed by treatment with NaOH for up to 0 02 N whereas treatment with 0 03 N NaOH reduced the activity and treatment with 0 04 and 0 05 N NaOH destroyed the activity of determinant *b* (Table 1) Anti Gc *b* apparently reacted with erythrocytes sensitized with samples of endotoxin treated with 0 04 and 0 05 N NaOH (see Fig 1) This activity must be due to a determinant group different from *b* The activity disappeared after digestion of the 0 04 and the 0 05 N NaOH treated preparations with pronase prior to sensitization of erythrocytes but not after periodate oxidation

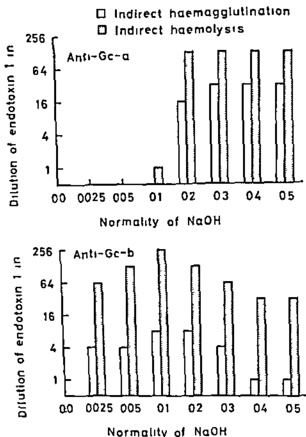


Fig 1

Erythrocyte sensitizing activity of aqueous ether endotoxin treated with various concentrations of NaOH Twofold dilutions of samples correspond ng to 1 mg per ml of endotoxin (ordinate) were used for sensitization Tested with 8 haemagglutinating units of anti Gc *a* (above) and anti Gc *b* (below) sera

As a standard procedure preparations sensitizing erythrocytes with determinant *a* were prepared as follows The endotoxin was treated with 0 04 N NaOH at 37 C for 16 hrs followed by neutralization with HCl and dialysis against phosphate buffered saline The protein component was then digested with pronase Four times the least amount of antigen which sensitized erythrocytes to maximal agglutination titres with antisera was employed for sensitization Thus from 125 to 250 µg of endotoxin was needed for sensitization of one ml of a one per cent suspension of erythrocytes Anti Gc *a* but not anti Gc *b* agglutinated the sensitized erythrocytes

Preparations sensitizing erythrocytes with determinant *b* were produced as follows The endotoxin was treated with 0 008 N NaOH at 37 C for 18 hrs followed by neutralization with HCl The preparation was then treated with periodate to destroy determinant *a* dialysed

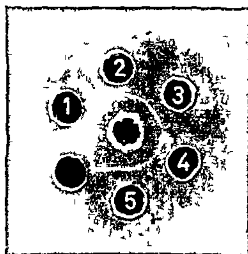


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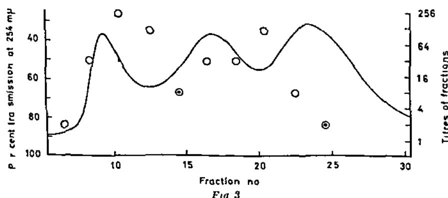


Fig 3

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—— Per cent transmission at 254 mμ
Titres of the antibodies to determinant *a*
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Anti SRE and anti Gc sera were subjected to gel filtration on Sephadex G 200 columns The haemagglutination titres of the fractions of the anti Gc serum were determined results are shown in Fig 3 The titres of the unfractionated antiserum were 512 and 1024 using the *a* and the *b* determinants respectively Antibodies to determinant *a* and to determinant *b* were found in the same fractions Treatment with mercaptoethanol completely destroyed the activity of fractions 6 to 10 but not the activity of fractions 11 to 24 Essentially the same results were obtained by Sephadex filtration of the anti SRE serum The results show that these antisera contained both γM and γG globulin antibodies to the determinants *a* and *b*

DISCUSSION

Endotoxin prepared from a *N gonorrhoeae* strain by extraction with aqueous ether required treatment with heat or alkali prior to sensitization of erythrocytes Phenol water lipopolysaccharides from gonococci (10) and other Gram negative cocci (6 7) as well as enterobacterial endotoxins (13) require similar treatment for sensitization While the effect on the endotoxin of such treatment was not investigated in the present study it has been correlated with the removal of some of the fatty acids or of O acetyl moieties (1 5)

Using different concentrations of NaOH for treatment of the endotoxin prior to sensitization it was shown that the availability of both the carbohydrate determinant (*a*) and the protein determinant (*b*) for their respective antibodies was affected

Anti Gc *b*, devoid of antibodies to determinant *a* reacted with erythrocytes sensitized with any of the alkali treated samples of endotoxin (Fig 1) However only samples treated with low concentrations of NaOH 0 03 N or less had the capacity to sensitize erythrocytes with determinant *b* since the antibody neutralizing ability of this determinant was destroyed by treatment with 0 04 or 0 05 N NaOH This means that the reaction of anti Gc *b* with erythrocytes sensitized with the 0 04 or 0 05 N NaOH treated preparations was due to a determinant group different from *b* probably one of protein nature It is possible that this determinant was made accessible by the alkali treatment

Endotoxin treated with 0 02 N or higher concentrations of NaOH sensitized erythrocytes for agglutination with antibodies to determinant *a* (Fig 1) Erythrocytes sensitized with samples of endotoxin treated with 0 01 N NaOH or less were not agglutinated Furthermore the results of the indirect haemolysis test the anti γ globulin test and the absorption experiment showed that the determinant *a* antibodies did not combine with these erythrocytes On the other hand the sensitized erythrocytes induced the formation in rabbits of antibodies to both *a* and *b* and therefore carried both the determinants Presumably some hindrance prevented the attachment of the *a* determinant antibodies

to erythrocytes sensitized with endotoxin which had been treated with low concentrations of NaOH The mechanism underlying this hindrance is not known

As indicated by results reported previously (10) erythrocytes sensitized with all ali extracted endotoxin acquire the specificity of determinant *b* Investigations have shown that anti Gc *b* but not anti Gc *a* combines with erythrocytes sensitized with the alkali endotoxin (*unpublished data*) However it has been shown by immunization experiments that during incubation with all ali extracted endotoxin both the determinants *a* and *b* adhered to the erythrocytes (9 10)

Due to the sensitizing properties of the aqueous ether endotoxin test systems for each of the determinants *a* and *b* could be developed This has been of great importance in the study of the endotoxin

Immunization of rabbits with erythrocytes sensitized with the aqueous ether endotoxin induced the formation of antibodies which could be detected by indirect haemagglutination and haemolysis precipitation complement fixation and bacterial agglutination tests This means that the antigenic determinants which attach to erythrocytes during incubation with the endotoxin have the capacity to participate in the various test systems Rabbit antibodies to the determinants *a* and *b* belong both to γM and to γG globulins analogous to rabbit antibodies to enterobacterial endotoxins (O antigens) (15)

Anti SRE and each of the specifically absorbed antisera (anti SRE *a* anti SRE *b*) formed one precipitation line against a suspension of bacterial cells These lines showed a reaction of identity in spite of the fact that determinant *a* and determinant *b* have different specificity However these determinants belong to one and the same molecular complex (11) a fact which might explain the precipitation pattern (14) On the other hand one or more determinants different from *a* and *b* may be involved in the precipitation reaction

The determinant groups present in the aqueous ether endotoxin function as agglutinogens in heated gonococci Using the slide agglutination test it has been observed that neither antiserum to the endotoxin (9) nor to heated gonococci (2) agglutinated live bacteria Therefore the tube agglutination technique was resorted to in this study It was however not possible to decide whether antibodies to the endotoxin have the capacity to agglutinate live gonococci since normal rabbit sera agglutinated live gonococci to comparatively high titres when this technique was used This agrees with the findings made by others (for references see 16) that sera from non immunized rabbits may contain antibodies reacting with gonococcal antigens

SUMMARY

Heat or alkali treatment of the aqueous ether endotoxin was necessary prior to sensitization of erythrocytes Both the carbohydrate and the

protein determinant of the endotoxin complex adsorbed to the ery throcytes However the different concentrations of sodium hydroxide used for treatment of the endotoxin prior to sensitization affected the availability of the carbohydrate and the protein determinant for their respective antibodies Techniques for sensitization of erythrocytes with each of these determinants are described

Antibodies to the endotoxin could be detected by indirect haemag glutination and haemolysis precipitation complement fixation and bacterial agglutination tests Rabbit antiserum to the endotoxin formed one precipitation line against whole gonococci Antibodies to the endo toxin belonged to γM and to γG globulins

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SEROLOGICAL CROSS REACTIONS OF AQUEOUS ETHER EXTRACTED ENDOTOXIN FROM *NEISSERIA GONORRHOEAE* STRAINS

By

J A Maeland

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The results of chemical and serological analyses of endotoxin obtained by extraction of a *N gonorrhoeae* strain (strain 8551) with aqueous ether have been reported previously (18 19) The endotoxin was found to be composed of lipid carbohydrate and protein the latter being the major constituent (18) One antigenic determinant is attached to the carbohydrate component (determinant *a*) and another to the protein component (determinant *b*) (17) Techniques for sensitization of erythrocytes with each of these determinants have been described (19)

Aqueous ether endotoxin from three strains of gonococci has been analysed chemically and serologically particularly with respect to serological cross reactivity of the *a* and *b* determinants Other strains of gonococci and of Gram negative cocci belonging to other species have been tested for serological cross reactivity with the *a* and *b* determinants from the three strains of gonococci The results are presented in this paper

MATERIALS AND METHODS

Strains

N gonorrhoeae strain 8551 has been characterized and described in earlier reports (14 15 16 17 18 19) Strains V and VII were obtained from the Neisseria Department Statens Seruminstitut Copenhagen Denmark These strains are designated the *reference strains* Rabbit antisera to each of these strains (anti Gc 8551 anti Gc V and anti Gc VII) were prepared as described previously (14) Other strains of gonococci included were obtained from the National Collection of Type Cultures London from the Neisseria Department Statens Seruminstitut Copenhagen and from the Department of Microbiology the Gade Institute Bergen Four strains of *N meningitidis* 11 strains of apathogenic *Neisseria* species one strain of *Mima polymorpha* and 2 strains of *Herellea* species were obtained from the National Collection of Type Cultures London One strain of oral *Veillonella* was isolated in the Department of Microbiology the Gade Institute Bergen All strains were preserved and stored by lyophilization

Cultivation and Harvesting

The *Veillonella* strain was cultured anaerobically in an atmosphere of 93 per cent of hydrogen and 7 per cent of carbon dioxide on Bacto Veillonella Agar (Difco) All

Antiserum to each of the strains agglutinated erythrocytes sensitized with both the homologous and heterologous *a* and *b* determinants (Table 2). Compared with the titres obtained with the homologous *a* determinants the titres of the antisera were generally lower when tested with the heterologous *a* determinants. The titres of the determinant *b* antibodies were the same irrespective of the strain from which the sensitizing antigen originated. Pre-immune sera diluted 1:16 gave no agglutination. Accordingly the *a* determinants as well as the *b* determinants from the reference strains cross-reacted serologically.

The results of tests for inhibition of haemagglutination in which 16 agglutinating units of the antisera were used are compiled in Table 3. The endotoxin preparations gave inhibition in the homologous but not in the heterologous test systems for the *a* determinants. Somewhat different results were obtained when 8 agglutinating units of the antisera were employed. Each endotoxin then inhibited the agglutination to some extent but not completely in heterologous systems for the *a* determinants with one exception. Endotoxin VII did not at all inhibit the agglutination with anti-Gc 8551 of erythrocytes sensitized with determinant *a* of endotoxin 8551.

TABLE 3

Minimal Inhibiting Dose (MID in μg) of Aqueous Ether Endotoxin from N. gonorrhoeae Strains 8551, X and VII Determined by Inhibition of Haemagglutination. Erythrocytes Sensitized with Determinant a *or Determinant* b *and Homologous Antisera were Used as Test Systems*

Preparation	MID in test system for Determinant *a* of endotoxin 8551	X	VII	Determinant *b* of endotoxin 8551	X	VII
Endotoxin 8551	12.50	>200	>200	12.50	12.50	12.50
Endotoxin X	>200	6.25	>200	12.50	6.25	6.25
Endotoxin VII	>200	>200	12.50	12.50	6.25	6.25

Each endotoxin inhibited about equally effectively in homologous and heterologous test systems for the *b* determinants (Table 3). Apart from a difference in minimal inhibiting dose the results obtained were the same no matter whether 8 or 16 agglutinating units of the antisera were employed.

The results of the inhibition tests indicate that the *b* determinants of the three endotoxins are identical with respect to specificity and that the *a* determinants are not identical.

Previous to the absorption experiments the minimal amount of endotoxin neutralizing the *b* determinant antibodies in 0.2 ml of undiluted homologous antiserum was determined. This amount of endotoxin was in excess of that required for neutralization of the *a* determinant antibodies owing to the higher titres of the antibodies to deter-

minant *b* The homologous anti Gc serum and each of the heterologous anti Gc sera 0 2 ml diluted 1 16 were absorbed with the estimated amount of endotoxin

Absorption with homologous and heterologous endotoxin removed equally effectively the antibodies to determinant *b* One absorption reduced the titres of the antisera from 2048 or more to less than 16 These findings agree with the results of the haemagglutination inhibition test and strongly indicate that the *b* determinants from the reference strains are identical

TABLE 4

Titres in the Indirect Haemagglutination Test of Rabbit Antisera to N gonorrhoeae Strains 8551 V and VII Absorbed with Endotoxins

Antiserum	Absorbed with	Erythrocytes sensitized with determinant *a* of endotoxin 8551	V	VII
Anti Gc 8551	Endotoxin 8551	<16	<16	<16
	V	256	<16	<16
	VII	512	*512*	<16
Anti Gc V	Endotoxin 8551	<16	*1024*	<16
	V	<16	<16	<16
	VII	256	*1024*	<16
Anti Gc VII	Endotoxin 8551	<16	*512*	*256*
"	V	<16	<16	*128*
"	VII	<16	<16	<16

The titres of the unabsorbed antisera are shown in Table 2

The effect of the absorptions on the titres of the *a* determinant antibodies is seen in Table 4 Absorption of homologous antiserum removed all the antibodies to determinant *a* Absorption of heterologous antiserum removed the antibodies cross reacting with the *a* determinant used for absorption but *a* determinant antibodies of other specificities were left unabsorbed indicating multispecificity of the component hitherto called determinant *a* The cross reactions between anti Gc 8551 and determinant *a* of endotoxin VII and between anti Gc VII and determinant *a* of endotoxin 8551 were eliminated by absorption with any of the endotoxins Therefore these cross reactions must be due to an antigenic specificity common to the reference strains Results similar to those shown in Table 4 were obtained by absorption with phenol water lipopolysaccharide or lyophilized bacteria

From Table 4 it is apparent that the various cross absorbed antisera and the sensitized erythrocytes which were agglutinated by these sera provided 9 test systems for haemagglutination inhibition experiments The endotoxins were examined for inhibition in each of these test systems The results presented further evidence of a multispecificity of the *a* determinants and showed most clearly the number of antigenic specificities (*a* factors) possessed by each of the reference

Antiserum to each of the strains agglutinated erythrocytes sensitized with both the homologous and heterologous a and b determinants (Table 2) Compared with the titres obtained with the homologous a determinants the titres of the antisera were generally lower when tested with the heterologous a determinants The titres of the determinant b antibodies were the same irrespective of the strain from which the sensitizing antigen originated Pre immune sera diluted 1 16 gave no agglutination Accordingly the a determinants as well as the b determinants from the reference strains cross reacted serologically

The results of tests for inhibition of haemagglutination in which 16 agglutinating units of the antisera were used are compiled in Table 3 The endotoxin preparations gave inhibition in the homologous but not in the heterologous test systems for the a determinants Somewhat different results were obtained when 8 agglutinating units of the anti sera were employed Each endotoxin then inhibited the agglutination to some extent but not completely in heterologous systems for the a determinants with one exception Endotoxin VII did not at all inhibit the agglutination with anti Gc 8551 of erythrocytes sensitized with determinant a of endotoxin 8551

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Preparation	MID in test system for					
	Determinant a of endotoxin			Determinant b of endotoxin		
	8551	V	VII	8551	V	VII
Endotoxin 8551	12 50	>200	>200	12 50	12 50	12 50
Endotoxin V	>200	6 25	>200	12 50	6 25	6 25
Endotoxin VII	>200	>200	12 50	12 50	6 25	6 25

Each endotoxin inhibited about equally effectively in homologous and heterologous test systems for the b determinants (Table 3) Apart from a difference in minimal inhibiting dose the results obtained were the same no matter whether 8 or 16 agglutinating units of the antisera were employed

The results of the inhibition tests indicate that the b determinants of the three endotoxins are identical with respect to specificity and that the a determinants are not identical

Previous to the absorption experiments the minimal amount of endotoxin neutralizing the b determinant antibodies in 0 2 ml of undiluted homologous antiserum was determined This amount of endotoxin was in excess of that required for neutralization of the a determinant antibodies owing to the higher titres of the antibodies to deter

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	VII	512	512	<16
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Preparation	MID in test system for					
	Determinant *a* of endotoxin			Determinant *b* of endotoxin		
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Endotoxin 8551	12.50	>200	>200	12.50	12.50	12.50
Endotoxin X	>200	6.25	>200	12.50	6.25	6.25
Endotoxin VII	>200	>200	12.50	12.50	6.25	6.25

Each endotoxin inhibited about equally effectively in homologous and heterologous test systems for the *b* determinants (Table 3). Apart from a difference in minimal inhibiting dose the results obtained were the same no matter whether 8 or 16 agglutinating units of the antisera were employed.

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		8551	V	VII
Anti Gc 8551	Endotoxin 8551	<16	<16	<16
"	" V	256	<16	<16
	VII	512	512	<16
Anti Gc V	Endotoxin 8551	<16	1024	<16
	V	<16	<16	<16
	VII	256	1024	<16
Anti Gc VII	Endotoxin 8551	<16	512	256
	V	<16	<16	128
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other strains were cultured on placental broth agar as described previously (14) The washed bacteria were stored frozen or lyophilized

Extraction Methods

Crude aqueous ether extracts were prepared from 27 strains of gonococci and from 4 strains of meningococci as described earlier (16) The aqueous ether endotoxin from the N gonorrhoeae strains 8551 V and VII (endotoxin 8551 endotoxin V and endotoxin VII) was purified by treatment of the crude extracts with DNase and repeated washings of the endotoxin with distilled water (17)

Phenol water lipopolysaccharides from strains 8551 V and VII were prepared as described earlier (16)

Serological Methods

Preparation of sensiti ed erythrocytes carrying determinant *a* or determinant *b* was performed as described earlier (19)

The indirect haemagglutination test and the test for inhibition of haemagglutination were performed according to previous reports (14 16) Unless otherwise stated 8 agglutinating units of the antisera were employed in the inhibition tests The minimal inhibiting dose (MID) is defined as the least amount of the preparation (in μg) which completely inhibits agglutination

Absorption of antiserum was performed either with endotoxin or with wet or lyophilized bacteria suspended in 1/150 M phosphate buffered saline pH 7 2 Lyophilized bacteria which did not form homogeneous suspensions were digested with trypsin (Trypure Novo) at 37 C for 2 hrs with an enzyme to sub trate ratio of 1 50 The enzyme was thereafter inactivated at 100 C for 5 mins The digestion invariably led to homogeneous bacterial suspensions Trypsin digestion and heating are without effect on determinants *a* and *b* (16) The absorptions were carried out at 4 C for 18 hrs followed by centrifugation at 5000 × *g* for 20 mins

Chemical Analyses

Protein was estimated by the Folin Ciocalteu method according to *Lowry et al* (12) with bovine serum albumin as standard and *nitrogen* by the micro Kjeldahl method (11) *Neutral sugar* was determined by the sulphuric acid orcinol method (28) with glucose galacto e 1 1 as standard and *hexosamine* by the method of *Rondle & Morgan* (21) with glucosamine as standard *Heptose* was sought by the cysteine sulphuric acid reaction of *Dische* (8) *Total phosphorus* was determined by a modification of the method of *Fiske & Subbarow* (9 29) *Lipid* was determined spectrophotometrically as fatty acid ester groupings with tripalmitin as standard (22 25) *DNA* was estimated by the diphenylamine reaction of *Dische* (7) with DNA from calf thymus (Sigma Chemical Company) as standard

Paper chromatography for detection of sugar con tituents was performed as de s ribed earlier (18) The method included hydrolysis with 2 N H_2SO_4 at 100 C for 3 hrs neutralization with barium hydroxide centrifugation and lyophilization of the supernatant The sugars were then extracted with pyridine and subjected to circular paper chromatography in ethyl acetate pyridine water (40 11 6) The chromatograms were developed with the silver nitrate reagent of *Trevelyan et al* (26)

EXPERIMENTS AND RESULTS

Chemical Analyses of Endotoxin 8551 Endotoxin V and Endotoxin VII

The physicochemical properties of the three endotoxins were strikingly similar The preparations formed opalescent suspensions in water precipitated during treatment with DNase (pH 5 5) and were completely sedimented by centrifugation at 30 000 x *g* for 60 mins The lyophilized preparations were insoluble in water but dissolved readily in alkaline solutions

The quantitative chemical data a compiled in Table 1 Both the

Folin and the nitrogen values show that protein accounts for some 80 to 90 per cent of the preparations. The amounts of neutral sugar and hexosamine were small, less than 2.2 per cent of each. Only endotoxin VII contained more than 3 per cent of fatty acids. The data shown in Table 1 account for about 94 per cent of endotoxin 8551 and 88 and 92 per cent of endotoxin V and endotoxin VII respectively.

TABLE 1

Chemical Composition of Aqueous Ether Endotoxin from N. gonorrhoeae Strains 8551, V and VII (Per Cent of Dry Weight)

Preparation	N	Protein	P	Neutral sugar	Hexosamine	Lipid
Endotoxin 8551	14.10	88	0.35	1.80	1.12	3.00
Endotoxin V	12.60	82	0.30	2.16	1.00	2.60
Endotoxin VII	12.90	83	0.40	1.43	0.70	6.30

Glucose, galactose and glucosamine were detected by paper chromatography of the hydrolysates. The chromogens formed by the endotoxin preparations in the cysteine sulphuric acid reaction for heptoses gave nearly identical absorption curves. One absorption peak with maximum at 390 mμ was due to the hexoses and a second peak with maximum at 505 mμ showed the presence of heptose (8).

The diphenylamine reaction for DNA was negative with endotoxin V and endotoxin VII whereas endotoxin 8551 contained one per cent of DNA.

Serological Cross Reactions of Endotoxin 8551, Endotoxin V and Endotoxin VII

Both the *a* and *b* determinants of the endotoxin preparations were examined for cross reactivity in the indirect haemagglutination and the haemagglutination inhibition tests. The antisera were used unabsorbed or absorbed with endotoxin.

TABLE 2

Titres in the Indirect Haemagglutination Test of Rabbit Antisera to N. gonorrhoeae Strains 8551, V and VII

Antiserum	Erythrocytes sensitized with: Determinant *a* of endotoxin 8551	V	VII	Determinant *b* of endotoxin 8551	V	VII
Anti Gc-8551	1024	512	128	4096	4096	4096
Anti Gc V	512	2048	256	2048	2048	2048
Anti Gc VII	512	1024	1024	2048	2048	2048

Antiserum to each of the strains agglutinated erythrocytes sensitized with both the homologous and heterologous *a* and *b* determinants (Table 2). Compared with the titres obtained with the homologous *a* determinants the titres of the antisera were generally lower when tested with the heterologous *a* determinants. The titres of the determinant *b* antibodies were the same irrespective of the strain from which the sensitizing antigen originated. Pre-immune sera diluted 1:16 gave no agglutination. Accordingly, the *a* determinants as well as the *b* determinants from the reference strains cross-reacted serologically.

The results of tests for inhibition of haemagglutination in which 16 agglutinating units of the antisera were used are compiled in Table 3. The endotoxin preparations gave inhibition in the homologous but not in the heterologous test systems for the *a* determinants. Somewhat different results were obtained when 8 agglutinating units of the antisera were employed. Each endotoxin then inhibited the agglutination to some extent but not completely in heterologous systems for the *a* determinants with one exception. Endotoxin VII did not at all inhibit the agglutination with anti Gc 8551 of erythrocytes sensitized with determinant *a* of endotoxin 8551.

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Preparation	MID in test system for					
	Determinant *a* of endotoxin			Determinant *b* of endotoxin		
	8551	X	VII	8551	X	VII
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Endotoxin VII	>200	>200	12.50	12.50	6.25	6.25

Each endotoxin inhibited about equally effectively in homologous and heterologous test systems for the *b* determinants (Table 3). Apart from a difference in minimal inhibiting dose the results obtained were the same no matter whether 8 or 16 agglutinating units of the antisera were employed.

The results of the inhibition tests indicate that the *b* determinants of the three endotoxins are identical with respect to specificity and that the *a* determinants are not identical.

Previous to the absorption experiments the minimal amount of endotoxin neutralizing the *b* determinant antibodies in 0.2 ml of undiluted homologous antiserum was determined. This amount of endotoxin was in excess of that required for neutralization of the *a* determinant antibodies owing to the higher titres of the antibodies to deter-

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	V	256	<16	<16
	VII	512	512	<16
Anti Gc V	Endotoxin 8551	<16	1024	<16
	V	<16	<16	<16
	VII	256	1024	<16
Anti Gc VII	Endotoxin 8551	<16	512	256
	V	<16	<16	128
"	VII	<16	<16	<16

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strains Table 5 shows the results obtained in the 6 test systems necessary for the demonstration of these factors The agglutination with unabsorbed anti Gc VII of erythrocytes sensitized with determinant a of endotoxin 8551 was inhibited by each of the endotoxins Therefore this inhibition was due to a factor shared by the endotoxins This factor is designated a_1 Either one or two of the endotoxin preparations gave inhibition in the 5 remaining test systems reflecting a factors possessed by only one of the preparations or factors shared by two of the preparations Only strain V possessed the factor called a Another factor called a_3 was shared by strain V and strain VII Only strain VII carried the factor called a_4 and factor a_5 occurred only in strain 8551 Strains 8551 and V had in common the factor called a_6 Since the b determinants of the reference strains have identical specificity the tentative antigenic formulas are as follows Endotoxin 8551 $ba_{1, 5, 6}$ Endotoxin V $ba_{1, \ldots, 3, 6}$ Endotoxin VII $ba_{1, 3, 4}$ Endotoxin 8551 and endotoxin VII have in common only factor a_1 whereas endotoxin V has two a factors in common with each of the other preparations Each endotoxin contained one a factor which was not present in the other preparations

It was found that most anti Gc 8551 sera contained antibodies of very low titres to factor a_1 Only one of 5 different sera tested for agglutination of erythrocytes sensitized with determinant a of endotoxin VII (a_1) gave a titre of 128 whereas the titres of the remaining sera varied from 16 to 32 Anti Gc V serum contained no antibodies to factor a_3 Nevertheless both the absorption experiments and the inhibition tests have shown that endotoxin V possessed factor a_3

Distribution of the a *Factors and of Determinant* b *among Gonococci and Gram Negative Cocci of other Species*

Samples of 200 and 100 µg of crude aqueous ether endotoxin prepared from 24 strains of gonococci and 4 strains of meningococci were

TABLE 5

Minimal Inhibiting Dose (MID in µg) of Aqueous Ether Endotoxin from N gonorrhoeae Strains 8551 V and VII Determined by Inhibition of Haemagglutination with Unabsorbed and Absorbed Antisera.

Test system made up of: Anti-Gc	Determinant a of	MID of endotoxin: 8551	V	VII	Factor designation
VII unabs.	End -8551	6.25	6.25	12.50	a_1
V abs. end. 8551	End V	[illegible]	3.12	[illegible]	a
VII abs. end. 8551	End. V	[illegible]	1.56		a_3
VII abs. end. V	End. VII	[illegible]	>200		a
-8551 abs. end. V	End.	12	>200		a_5
-8551 abs. end. VII	End. V	25	[illegible]		a

Anti-Gc Anti erum [illegible]

TABLE 6

Distribution of Determinant b and of the Factors of Determinant a in N gonorrhoeae and N meningitidis Strains Determined by Inhibition of Haemagglutination with Aqueous Ether Extracts

Strains	Factors of determinant a: a_1	a_2	a_3	a_4	a_5	a_6	Determinant b
N gonorrhoeae							
SS 119624 SS 117573 SS 120469	+	—	—	—	—	+	+
CI 8116 SS VII	+	—	+	+	—	—	+
CI 6976	+	—	—	+	—	+	+
CI 7471 CI 8551 SS 120709 SS 126779	+	—	—	—	+	+	+
CI 7174 CI 8172 CI 9142 SS V SS 124392 SS 126492	+	+	+	—	—	+	+
CI 6115 GI 6526 SS III	+	+	—	—	+	+	+
SS 126676	+	—	+	+	—	+	+
SS 117579	+	—	—	+	+	+	+
NCTC 8?0	+	+	+	+	—	+	+
SS 1?6582 SS 126773 SS 126880 SS 126879	+	+	+	—	+	+	+
NCTC 7119	+	+	—	+	+	+	+
N meningitidis							
group A NCTC 10025	—	—	—	—	—	+	+
group B NCTC 10026	+	—	—	—	—	+	+
group C NCTC 8554	+	—	—	—	—	—	+
group D NCTC 6457	—	—	—	—	—	—	+

SS The Neisseria Department Statens Seruminstitut Copenhagen
GI The Department of Microbiology the Gade Institute Bergen
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+ and — indicate presence or absence of the *a* factors and determinant *b*

strains. Table 5 shows the results obtained in the 6 test systems necessary for the demonstration of these factors. The agglutination with unabsorbed anti Gc VII of erythrocytes sensitized with determinant a of endotoxin 8551 was inhibited by each of the endotoxins. Therefore this inhibition was due to a factor shared by the endotoxins. This factor is designated a_1. Either one or two of the endotoxin preparations gave inhibition in the 5 remaining test systems reflecting a factors possessed by only one of the preparations or factors shared by two of the preparations. Only strain V possessed the factor called a. Another factor called a_3 was shared by strain V and strain VII. Only strain VII carried the factor called a_4 and factor a_5 occurred only in strain 8551. Strains 8551 and V had in common the factor called a_6. Since the b determinants of the reference strains have identical specificity the tentative antigenic formulas are as follows: Endotoxin 8551: $ba_{1,5,6}$; Endotoxin V: $ba_{1,\ 3,6}$; Endotoxin VII: $ba_{1,3,4}$. Endotoxin 8551 and endotoxin VII have in common only factor a_1, whereas endotoxin V has two a factors in common with each of the other preparations. Each endotoxin contained one a factor which was not present in the other preparations.

It was found that most anti Gc 8551 sera contained antibodies of very low titres to factor a_1. Only one of 5 different sera tested for agglutination of erythrocytes sensitized with determinant a of endotoxin VII (a_1) gave a titre of 128, whereas the titres of the remaining sera varied from 16 to 32. Anti Gc V serum contained no antibodies to factor a_3. Nevertheless both the absorption experiments and the inhibition tests have shown that endotoxin V possessed factor a_3.

Distribution of the a Factors and of Determinant b *among Gonococci and Gram Negative Cocci of other Species*

Samples of 200 and 100 μg of crude aqueous ether endotoxin prepared from 24 strains of gonococci and 4 strains of meningococci were

TABLE 5

Minimal Inhibiting Dose (MID in μg) of Aqueous Ether Endotoxin from N. gonorrhoeae *Strains 8551, V and VII Determined by Inhibition of Haemagglutination with Unabsorbed and Absorbed Antisera*

Test system made up of: Anti Gc	Determinant a of	MID of endotoxin: 8551	V	VII	Factor designation
VII unabs	End. 8551	6.25	6.2	12.50	a_1
V abs end. 8551	End. V	>200	3.12	>200	a
VII abs end. 8551	End. V	>200	1.56	12.50	a_3
VII abs end. V	End. VII	>200	>200	12.50	a_4
8551 abs end. V	End. 8551	3.12	>200	>200	a
8551 abs end. VII	End. V	6.25	12.50	>200	a_6

Anti Gc: Antiserum to whole gonococci

TABLE 6

Distribution of Determinant b and of the Factors of Determinant a in N gonorrhoeae and N meningitidis Strains Determined by Inhibition of Haemagglutination with Aqueous Ether Extracts

Strains	Factors of determinant a						Determinant b
	a_1	a	a_3	a_4	a_5	a_6	
N gonorrhoeae							
SS 119694 SS 117573 SS 120469	+	—	—	—	—	+	+
GI 8116 SS VII	+	—	+	+	—	—	+
CI 6976	+	—	—	+	—	+	+
GI 7471 GI 8551 SS 120709 SS 126772	+	—	—	—	+	+	+
CI 7174 CI 8172 CI 9142 SS V SS 124392 SS 126492	+	+	+	—	—	+	+
GI 6115 CI 6576 SS III	+	+	—	—	+	+	+
SS 120676	+	—	+	+	—	+	+
SS 117579	+	—	—	+	+	+	+
NCTC 6890	+	+	+	+	—	+	+
SS 126582 SS 126773 SS 126890 SS 126899	+	+	+	—	+	+	+
NCTC 7129	+	+	—	+	+	+	+
N meningitidis							
group A NCTC 10025	—	—	—	—	—	+	+
group B NCTC 10026	+	—	—	—	—	+	+
group C NCTC 8554	+	—	—	—	—	—	+
group D NCTC 6457	—	—	—	—	—	—	+

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strains Table 5 shows the results obtained in the 6 test systems necessary for the demonstration of these factors The agglutination with unabsorbed anti Gc VII of erythrocytes sensitized with determinant a of endotoxin 8551 was inhibited by each of the endotoxins Therefore this inhibition was due to a factor shared by the endotoxins This factor is designated a_1 Either one or two of the endotoxin preparations gave inhibition in the 5 remaining test systems reflecting a factors possessed by only one of the preparations or factors shared by two of the preparations Only strain V possessed the factor called a Another factor called a_3 was shared by strain V and strain VII Only strain VII carried the factor called a_4 and factor a_5 occurred only in strain 8551 Strains 8551 and V had in common the factor called a_6 Since the b determinants of the reference strains have identical specificity the tentative antigenic formulas are as follows Endotoxin 8551 $ba_{1,5,6}$ Endotoxin V $ba_{1,3,6}$ Endotoxin VII $ba_{1,3,4}$ Endotoxin 8551 and endotoxin VII have in common only factor a_1 whereas endotoxin V has two a factors in common with each of the other preparations Each endotoxin contained one a factor which was not present in the other preparations

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VII abs end V	End VII	>200	>200	12.50	a_4
8551 abs end V	End 8551	3.12	>200	>200	a
8551 abs end VII	End V	6.25	12.50	>200	a_6

Anti Gc Antiserum to whole gonococci

To test for cross reaction with bacteria other than gonococci and meningococci 16 agglutinating units of the various antisera were absorbed with 1 5 mg of lyophilized bacteria and then tested for agglutination of sensitized erythrocytes About 0 15 mg of lyophilized gonococci of the homologous strains was required for absorption of 16 units of these antibodies

None of the bacteria listed in Table 7 absorbed the antibodies to the factors a_1 a a_3 and a or the antibodies to determinant *b* *N canis* and *N denitrificans* absorbed the antibodies to a_4 and a_6 whereas none of the other strains absorbed the antibodies to a_4 The antibodies to a_6 were also removed by absorption with *N catarrhalis* (one strain) *N pharyngis* (one strain) *N flavescens* (2 strains) and the *Veillonella* strain The results demonstrate some serological cross reactivity between the carbohydrate component of endotoxins from gonococci and apathogenic *Neisseria*

DISCUSSION

Aqueous ether endotoxin prepared from the reference gonococcal strains 8551 V and VII has been analysed and compared chemically and serologically The endotoxin preparations were essentially similar with respect to physical properties and chemical composition and proved to be complexes of lipid carbohydrate and protein While protein constituted from 80 to 90 per cent of the endotoxins the carbohydrate components composed of glucose galactose glucosamine and heptose amounted to less than 5 per cent With the reservation that minute amounts of other sugars may have escaped detection it is concluded that the endotoxins of the reference strains belong to the same chemotype *i e* have the same qualitative sugar composition

Determinant b

The protein determinant (*b*) of endotoxins prepared from the reference strains cross reacted serologically and haemagglutination inhibition and absorption experiments presented evidence that these determinants are identical Furthermore all the other strains of gonococci examined (64 strains) and of meningococci (4 strains) cross reacted with determinant *b* of endotoxin 8551 On the other hand strains of Gram negative cocci belonging to other species showed no cross reactivity These findings strongly indicate that determinant *b* is a group reactive antigen common to gonococci and meningococci Therefore tests for cross reactivity with determinant *b* may offer a supplementary method for differentiation of gonococci and meningococci on the one hand and other Gram negative cocci on the other

A close antigenic relationship between gonococci and meningococci has been demonstrated by several investigators *Wilson* (27) and *Deacon et al* (6) using bacterial agglutination and fluorescent anti

examined for inhibition of haemagglutination in the various test systems for a factors shown in Table 5 The preparations were also tested for inhibition of haemagglutination in the test system for determinant b of endotoxin 8551

It appears from Table 6 that all gonococcal strains contained at least two of the a factors Although the factors occurred in different combinations 22 out of the 27 gonococcal strains including the reference strains belonged to groups comprising from 2 to 6 strains carrying identical factors While all strains of gonococci possessed factor a_1 factor a_6 was also found in all strains except strain VII and another strain with identical a factors ($ba_{1\ 3\ 4}$) Four strains possessed the same factors as strain 8551 ($ba_{1,\ 6}$) and 6 strains the factors of strain X ($ba_{1\ 3\ 6}$)

The a factors were however not restricted to gonococci since groups B and C of the meningococcal strains examined contained factor a_1 and groups A and B of the meningococcal strains contained factor a_6

All endotoxin preparations from the strains listed in Table 6 including those from meningococci gave inhibition in the test system for determinant b Anti Gc 8551 0 1 ml diluted 1 16 was absorbed with approximately 200 mg of wet cells of 40 other strains of gonococci One absorption with each of these strains removed the antibodies to determinant b indicating identity or close similarity of the b determinants

The serological activity of the endotoxin preparations from meningococci and from 5 strains of gonococci was compared in the test systems for a factors and for determinant b The minimal inhibiting doses varied between 1 56 and 25 μg

TABLE 7

Distribution of the Factors a_1 and a_6 in Gram Negative Cocci other than Gonococci and Meningococci Determined by Absorption of Antisera with Bacterial Cells

Strains tested		Factors a_1	Factors a_6
N catarrhalis	NCTC 3622	—	—
N catarrhalis	4103	—	+
N pharyngis	4590	—	—
N pharyngis	4591	—	+
N flavescens	8263	—	+
N flavescens	3191	—	+
N cuniculi	10297	—	—
N canis	10296	+	+
N caviae	10293	—	—
N denitrificans	10295	+	+
N haemolysans	10243	—	—
Mima polymorpha	797[illegible]	—	—
Herellea vaginalis	7844	—	—
Herellea vaginalis	7250	—	—
Veillonella		—	+

+ and — indicate presence or absence of the a factors

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DISCUSSION

Aqueous ether endotoxin prepared from the reference gonococcal strains 8551 X and XII has been analysed and compared chemically and serologically The endotoxin preparations were essentially similar with respect to physical properties and chemical composition and proved to be complexes of lipid carbohydrate and protein While protein constituted from 80 to 90 per cent of the endotoxins the carbohydrate components composed of glucose galactose glucosamine and heptose amounted to less than 5 per cent With the reservation that minute amounts of other sugars may have escaped detection it is concluded that the endotoxins of the reference strains belong to the same chemotype *i e* have the same qualitative sugar composition

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A close antigenic relationship between gonococci and meningococci has been demonstrated by several investigators *Wilson* (27) and *Deacon et al* (6) using bacterial agglutination and fluorescent anti

body techniques respectively have shown that gonococci and meningococci share heat stable antigens Serological cross reactivity of toxic materials from gonococci and meningococci has been reported (1 2 23) *Reyn* (20) *using the complement fixation test observed a thermostable* common gonococcus antigen In view of the fact that determinant *b* is thermostable (16) and is closely associated with the endotoxin (17) it seems highly probable that this determinant is identical to or part of the cross reacting antigens described by these authors *Chanarin* (3) prepared an erythrocyte sensitizing antigen by extraction of *gonococci* with alkali and *found* that this antigen was type specific (type I and type II) In all 59 of 67 strains belonged to type I 8 strains to type II Meningococci cross reacted with the type I antigen It has previously been shown that erythrocytes sensitized with the alkali extract acquire the specificity of determinant *b* (16 19) a finding which indicates that determinant *b* is identical with *Chanarin*s type I antigen No type II antigen has been detected in this study

The author has previously reported that 55 per cent of blood donor sera contained antibodies to erythrocytes sensitized with the alkali extract (14) *i e antibodies to determinant b Contact with meningo*cocci may have induced the formation of the determinant *b* antibodies in many of the healthy individuals

Antigenic determinants of endotoxins (O antigens) are usually of carbohydrate nature (13) although determinants of protein nature have also been reported (10) However both the present study and results reported previously (14 15 16 17 19) have clearly shown that the protein component plays an important part in the serology of the endotoxin from gonococci

Determinant a

The *a* determinants from the reference strains cross reacted serologically but were not identical since the preparations failed to inhibit completely the haemagglutination in heterologous test systems for the *a* determinants The results of cross absorptions of antisera and of haemagglutination inhibition tests with cross absorbed sera (cfr Table 4 and 5) showed that each *a* determinant comprises several antigenic specificities (*a* factors) Six *a* factors (a_1 a_6) were revealed The cross reactions were thus due to *a* factors shared by the cross reacting endotoxins The possibility that some of the *a* factors consist of more than one antigenic specificity cannot be excluded Moreover other *a* factors may well exist within the species *N gonorrhoeae* in addition to the *a* factors described In order to settle these matters experiments with additional reference strains and antisera to these strains are required

As the effect of cross absorption with phenol water lipopolysaccharides or lyophilized bacteria was the same as that of cross absorp

tion with aqueous ether endotoxin the latter preparation must contain all the antigenic specificities which belong to the carbohydrate component of gonococcal endotoxins

The a factors of each strain of gonococci certainly belong to one and the same molecular complex analogous to the O factors of *Salmonella* endotoxins (13) It was found that the endotoxins prepared from the reference strains belonged to the same chemotype with regard to sugar constituents *Salmonella* and *Escherichia* O antigens belonging to the same chemotype may include several serotypes (13 30) *Lüderitz et al* (13) suggested that in these instances the same sugars are linked in different ways which would explain the difference in specificity The results of tests for inhibition of haemagglutination with mono and disaccharides have indicated that the difference in specificity of the various a factors of gonococci can be explained in the same way As reported earlier galatose and lactose inhibited the reaction of anti Gc 8551 with determinant a of endotoxin 8551 (18) Investigations currently in progress have shown that galactose and lactose also inhibit the combination of anti Gc V with determinant a of endotoxin V but not the combination of anti Gc VII with determinant a of endotoxin VII Further investigations along these lines will probably provide valuable information about the structures which determine the specificity of the various a factors

The six a factors demonstrated in this study were found to be widely distributed among strains of gonococci Although the combination of factors varied considerably 22 out of 27 strains could be divided into 6 groups each group comprising from 2 to 6 strains with identical factors Classification of gonococci based on the a factors thus seems possible but further investigations are needed to evaluate the practical application of these findings All strains of gonococci examined contained factor a_1 which therefore may be common to all gonococci However this factor is not limited to the species *N gonorrhoeae* since 2 out of 4 strains of meningococci examined cross reacted with factor a_1 Moreover 2 strains of meningococci cross reacted with factor a_6 The close antigenic relationship between gonococci and meningococci is also apparent from these cross reactions

Some strains of Gram negative cocci other than gonococci and meningococci cross reacted with factor a factor a or both Accordingly some antigenic relationship between gonococci and apathogenic *Neisseria* species exists in agreement with observations made by others (4 5 6 23 24)

To my knowledge there are no published reports on multispecificity of the carbohydrate component of endotoxins from *Neisseria* species It is obvious that the endotoxin of gonococci is a highly complex substance which requires further investigations for its characterization

SUMMARY

Aqueous ether endotoxin prepared from three strains of gonococci contained from 80 to 90 per cent of protein and small amounts of carbohydrate and lipid Each endotoxin contained glucose galactose glucosamine and heptose

Tests for serological cross reactivity of the endotoxins presented evidence that the antigenic determinant of carbohydrate nature (*a*) was multispecific Six antigenic specificities (*a* factors) were demonstrated Endotoxin prepared from other strains of gonococci cross reacted with several of the *a* factors Some strains of meningococci and other Gram negative cocci cross reacted with one or two of the *a* factors

The antigenic determinant which belongs to the protein component (*b*) of the aqueous ether endotoxin was found to be a group reactive antigen common to gonococci and meningococci Strains of Gram negative cocci belonging to other species gave no cross reaction with this determinant

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MATERIALS AND METHODS

Animals The mice were highly inbred female C_3H mice raised at our institute The rabbits were non inbred white rabbits

Antilymphocyte serum ALS was prepared from a rabbit by i v injection of 200 × 10^6 C_3H lymph node cells suspended in phosphate buffered saline (PBS) This was repeated three times at weekly intervals and one week after the last injection the animal was bled This serum was used for the preliminary experiment The rabbit was boosted and rebled twice and the serum from the second rebleeding was used in the experiments proper The sera were inactivated and kept at −20 C until required

Normal rabbit serum NRS was obtained in the same way except that the animal was injected with saline only

The sera were tested for haemagglutinins and leuco agglutinins against C_3H red and white cells and titres of 16 and 32 respectively were found in the first preparation of ALS while no such activity was found in the NRS

Virus The virus was a 10 per cent clarified homogenate of spleens harvested seven days after i p LCM infection of adult C_3H female mice

Virus titrations were carried out in ten fold dilutions in PBS of heparinized blood drawn from the retro orbital plexus Each dilution was injected into four ordinary Swiss mice The deaths occurring within 14 days were recorded and the titres calculated according to *Karber*s method (6) The titres are expressed as log 10 LD / 0 03 cc

CF antibodies were measured as described in a previous report (16)

Donor mice Mice which have nursed LCM infected offspring develop a life long immunity to reinfection and persisting CF antibodies (8) The spleens of such immune animals were used for transplantation Before transplantation the sera of the mice were titrated for CF antibodies and titres between 64 and 512 were found

Recipient mice Mice which are infected with >10 LD$_{50}$ LCM virus i p within 18 hours of birth develop a life long tolerant infection (7) In this laboratory these virus carrier mice are characterized by a constant viraemia with titres ≥ 2 3 CF antibodies <4 and no signs of disease Female virus carrier mice of this type were used as recipients of the syngeneic donor cells Before transplantation the viraemia was >3 0 in all recipients and the CF antibodies <4

Transplantation of cells Spleens from ten immune donors were excised under aseptic precautions cut into pieces and pressed through a stainless steel mesh into Hanks BSS with penicillin and streptomycin After careful mixing the cells were divided into three parts one containing 1/2 and the remaining two 1/4 of the original volume They were then washed three times in Hanks BSS and resuspended in different fluids The first portion (1/2 of the cells) was resuspended in 6 ml of undiluted ALS and incubated at 37 C for 1½ hours with frequent mixing No appreciable macroscopic or microscopic agglutination occurred during the incubation period Thereafter the cells were washed three times in Hanks BSS resuspended and counted after staining with eosin This revealed uptake of eosin by 35 per cent of the cells Finally 30 × 10^6 living cells were injected i p into the recipient virus carrier mice

The second portion of the cells (1/4 of the original volume) was treated identically except that they were incubated with 3 ml of undiluted NRS The counting this time revealed uptake of eosin by 25 per cent of the cells

The third portion containing the remaining 1/4 of the original immune cells was incubated with Hanks BSS and here the eosin uptake was 30 per cent

The percentage of cells which took up eosin in the three portions of cells was higher than is usually seen in our transplantation experiments (10–15 per cent) This was thought to be due to the relatively long in vitro processing

Both the second and the third part of cells were injected into virus carrier recipients in doses of 30 × 10^6 living cells per mouse

In all three groups of recipients the virus contents in the blood and the CF antibodies in the sera were measured individually at intervals

RESULTS

In a preliminary experiment a group of five LCM virus carrier mice was transplanted i p with 16 × 10 living ALS treated syngeneic im

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The Institute of Medical Microbiology University of Copenhagen Denmark

EFFECT OF ANTILYMPHOCYTE SERUM ON ADOPTIVE IMMUNIZATION OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS CARRIER MICE

By

CLAUS LUNDSTEDT

Received 14 v 69

The marked suppressive effect of antilymphocyte serum (ALS) on cell mediated immunity has been demonstrated several times recently (10 11 18) This is in contrast to the almost complete absence of effect on the humoral antibody response especially on the secondary response (3 5) In vitro ALS is capable of exerting a complement dependent lymphocytotoxic effect or—in the absence of complement—a leuco agglutinating effect However these effects cannot always be related to the in vivo effect on for example skin grafts (4 9)

During the past few years evidence has been accumulating which indicates that the virus eliminating mechanism seems to be cell mediated in a least some virus infections (1 2 3 7 12 17) Thus it has been shown that adoptive immunization of tolerant lymphocytic choriomeningitis (LCM) virus carrier mice can be accomplished only by immunologically competent cells (normal or immune) and not by hyperimmune anti LCM serum (15) Furthermore in this laboratory we have been able to provoke a viraemia in LCM immune mice by a long ALS treatment without significantly affecting the content of complement fixing (CF) antibodies in the sera of these mice (17) The viraemia was thought to arise from the observed virus reservoir in the kidneys thymuses or lungs of such immune animals (14) Moreover rubella infected children have been shown to harbour virus in their naso pharynx for months whilst at the same time the serum contains neutralizing fluorescent haemagglutination inhibiting and CF antibodies (12)

The present experiments were therefore undertaken to throw further light upon the relative importance of cell mediated and humoral immunity in LCM infection The report describes the effect of ALS on the ability of syngeneic LCM immune spleen cells to confer adoptive immunization on LCM tolerant virus carrier mice

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MATERIALS AND METHODS

Animals The mice were highly inbred female C_3H mice raised at our institute The rabbits were non inbred white rabbits

Antilymphocyte serum ALS was prepared from a rabbit by i v injection of 200 × 10^6 C_3H lymph node cells suspended in phosphate buffered saline (PBS) This was repeated three times at weekly intervals and one week after the last injection the animal was bled This serum was used for the preliminary experiment The rabbit was boosted and rebled twice and the serum from the second rebleeding was used in the experiments proper The sera were inactivated and kept at —20 C until required

Normal rabbit serum NRS was obtained in the same way except that the animal was injected with saline only

The sera were tested for haemagglutinins and leuco agglutinins against C_3H red and white cells and titres of 16 and 32 respectively were found in the first preparation of ALS while no such activity was found in the NRS

Virus The virus was a 10 per cent clarified homogenate of spleens harvested seven days after i p LCM infection of adult C H female mice

Virus titrations were carried out in ten fold dilutions in PBS of heparinized blood drawn from the retro orbital plexus Each dilution was injected into four ordinary Swiss mice The deaths occurring within 14 days were recorded and the titres calculated according to *Kärber s* method (6) The titres are expressed as log 10 LD_{50}/ 0 03 cc

CF antibodies were measured as described in a previous report (16)

Donor mice Mice which have nursed LCM infected offspring develop a life long immunity to reinfection and persisting CF antibodies (8) The spleens of such immune animals were used for transplantation Before transplantation the sera of the mice were titrated for CF antibodies and titres between 64 and 512 were found

Recipient mice Mice which are infected with $>10\ LD_{50}$ LCM virus i p within 18 hours of birth develop a life long tolerant infection (7) In this laboratory these virus carrier mice are characterized by a constant viraemia with titres $\geq 2\ 3$ CF antibodies <4 and no signs of disease Female virus carrier mice of this type were used as recipients of the syngeneic donor cells Before transplantation the viraemia was $>3\ 0$ in all recipients and the CF antibodies <4

Transplantation of cells Spleens from ten immune donors were excised under aseptic precautions cut into pieces and pressed through a stainless steel mesh into Hanks BSS with penicillin and streptomycin After careful mixing the cells were divided into three parts one containing 1/2 and the remaining two 1/4 of the original volume They were then washed three times in Hanks BSS and resuspended in different fluids *The first portion (1/2 of the cells)* was resuspended in 6 ml of undiluted ALS and incubated at 37 C for 1¼ hours with frequent mixing No appreciable macroscopic or microscopic agglutination occurred during the *incubation period* Thereafter the cells were washed three times in Hanks BSS resuspended and counted after staining with eosin This revealed uptake of eosin by 35 per cent of the cells *Finally* 30×10^6 living cells were injected i p into the recipient virus carrier mice

The second portion of the cells (1/4 of the original volume) was treated identically except that they were incubated with 3 ml of undiluted NRS The counting this time revealed uptake of eosin by *25 per cent of the* cells

The third portion containing the remaining 1/4 of the original immune cells was incubated with Hanks BSS and *here the eosin* uptake was 30 per cent

The percentage of cells which took up eosin in the three portions of cells was higher than is usually seen in our *transplantation* experiments (10–15 per cent) This was thought to be due to the relatively long in vitro processing

Both the second and the third part of cells were injected into virus carrier recipients in doses of 30 × 10^6 living cells per mouse

In all three groups of recipients the virus contents in the blood and the CF antibodies in the sera were measured individually at intervals

RESULTS

In a preliminary experiment a group of five LCM virus carrier mice was transplanted i p with 16 × 10^6 living ALS treated syngeneic im

mune spleen cells isolated from four immune donors The cells had been incubated with 1 5 ml of ALS and washed as described

One mouse in the group died 12 days after transplantation and was never investigated A second mouse died after 40 days At autopsy both of these mice were found to have a thymoma A third mouse died after four months without any obvious reason but it must be noted that all the mice in this group were 8–9 months old at the time of transplantation

The four mice which were investigated all developed CF antibodies in titres initially varying between 512 and 2048 In the two long term survivors the titres gradually decreased to about 128 in the course of 2–3 months However the virus titres remained at virus carrier levels (i e ≥ 2 3) throughout the observation period which was 253 days in the case of mice surviving for the longest period

Mice in one control group comprising five LCM virus carrier mice received 16 × 16^6 living cells from the same pool of cells However these cells were treated with 1 5 ml of NRS In this group all the mice developed CF antibodies with titres between 1024 and 8192 stabilizing at about 1024 in 1–2 months The virus titres gradually declined in 1–2 months to titres $\leq$ 0 5 Thus a complete adoptive immunization was obtained One mouse died 78 days after transplantation

A second control group also consisted of five LCM tolerant animals which received 16 × 10^6 living cells from the same pool of cells as the experimental group But here the cells were incubated with Hanks BSS instead of ALS or NRS All five mice developed CF antibodies in titres between 2048 and 16 384 decreasing in 1–2 months to about 1024 In three of the mice the virus was eliminated from the blood to titres $\leq$ 0 5 and a complete adoptive immunization was obtained The remaining two however retained their viraemia on virus carrier level as in the experimental group This to some extent invalidated the results an it was thought to be due to the dose of transplanted cells (16 × 10^6) which might have been near the lower limit of that necessary to obtain a constant effect

The experiments were therefore repeated with the procedure as described in materials and methods The important alterations from the preliminary experiments were 1) the transplanted dose of cells was now 30 × 10^6 and 2) the recipient animals were only about three months old at the time of transplantation The results are shown in Table 1 and Figs 1 and 2

The experimental group consisted of ten female virus carrier mice which received ALS treated syngeneic LCM immune spleen cel As can be seen from Table 1 the results can be divided into groups Either the recipients developed CF antibodies (with riation in titres) and no major alteration in virus titres r adoptive immunization (seven mice nos 2 3 5 6 7 8 9) veloped CF antibodies which remained at a higher level f

TABLE 1

LCM Virus and CF Titre in Blood of LCM Virus Carrier Mice Transplanted with LCM Immune Syngeneic Cells

Animal groups	Days after transplantation: 0		15		41		77		113		166	
	Virus	CF	Virus	CF	Virus	CF	Virus	CF	Virus	CF	Virus	CF
Experimental group receiving ALS treated cells												
Mouse no 1	3.0	<4	2.3	512	1.5	256	≤0.5	256	1.0	256	≤0.5	512
2	ND	ND	3.5	16	3.0	8	3.5	4	3.0	4	3.5	<4
3	3.3	<4	2.0	256	3.5	128	3.8	32	3.5	32	2.0	64
4	3.0	<4	3.8	≥2048	2.5	1024	≤0.5	512	≤0.5	512	1.0	512
5	3.0	<4	2.5	512	3.3	8	4.0	4	2.8	<4	3.3	<4
6	3.0	<4	3.5	1024	3.3	32	3.5	16	3.3	16	4.0	16
7	≥3.5	<4	2.3	≥2048	2.5	≥2048	1.8	512	3.3	64	4.0	32
8	3.3	<4	2.8	512	2.5	128	3.3	64	4.3	64	3.0	32
9	≥3.5	<4	3.3	1024	2.3	512	2.0	1024	1.5	512	3.3	512
10	3.3	<4	2.5	≥2048	1.3	1024	≤0.5	512	≤0.5	1024	≤0.5	≥2048
Control group receiving NRS treated cells	5/5	5/5	4/4	4/4	3/3	3/3	3/3	2/3	3/3	2/3	2/3	2/3
	≥3.0	<4	≤0.5	≥2048	≤0.5	≥2048	≤0.5	512	≤0.5	≥2048	≤0.5	≥2048
Control group receiving Hanks BSS treated cells	5/5	5/5	4/4	3/4†	4/4	4/4	4/4	2/4	4/4	2/4	3/4§	2/4
	≥3.3	<4	≤0.5	≥2048	≤0.5	≥2048	≤0.5	≥2048	≤0.5	512	≤0.5	≥2048

ND = Not Done   Remaining mouse ≥2048   Remaining mouse/mice 1024   Remaining mouse 1.0   † Remaining mouse Not done   § Remaining mouse 2.0

mune spleen cells isolated from four immune donors The cells had been incubated with 1 5 ml of ALS and washed as described

One mouse in the group died 12 days after transplantation and was never investigated A second mouse died after 40 days At autopsy both of these mice were found to have a thymoma A third mouse died after four months without any obvious reason but it must be noted that all the mice in this group were 8-9 months old at the time of transplantation

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The experimental group consisted of ten female virus carrier mice which received ALS treated syngeneic LCM immune spleen cells i p As can be seen from Table 1 the results can be divided into two distinct groups Either the recipients developed CF antibodies (with great variation in titres) and no major alteration in virus titres i e a partial adoptive immunization (seven mice nos 2 3 5 6 7 8 9) or they developed CF antibodies which remained at a higher level for a longer

TABLE 1

LCM Virus and CF Titers in Blood of LCM Virus Carrier Mice Transplanted with LCM Immune Syngeneic Cells

Animal groups	Days after transplantation 0 Virus	0 CF	15 Virus	15 CF	41 Virus	41 CF	77 Virus	77 CF	113 Virus	113 CF	166 Virus	166 CF
Experimental group receiving ALS treated cells												
Mouse no 1	3.0	<4	2.5	512	1.5	256	≤ 0.5	256	1.0	256	≤ 0.5	512
2	ND	ND	3.5	16	3.0	8	3.5	4	3.0	4	3.5	<4
3	3.3	<4	2.0	256	3.5	128	3.8	32	3.5	32	2.0	64
4	3.0	<4	3.8	≥ 2048	2.5	1024	≤ 0.5	512	≤ 0.5	512	1.0	512
5	3.0	<4	2.5	512	3.3	8	4.0	4	2.8	<4	3.3	<4
6	3.0	<4	3.5	1024	3.3	32	3.5	16	3.3	16	4.0	16
7	≥ 3.5	<4	2.5	≥ 2048	2.5	≥ 2048	1.8	512	3.3	64	4.0	32
8	3.3	<4	2.8	512	2.5	128	3.3	64	4.3	64	3.0	32
9	≥ 3.5	<4	3.3	1024	2.3	512	2.0	1024	1.5	512	3.3	512
10	3.3	<4	2.5	≥ 2048	1.3	1024	≤ 0.5	512	≤ 0.5	1024	≤ 0.5	≥ 2048
Control group receiving NRS treated cells	5/5 ≥ 3.0	5/5 <4	4/4 ≤ 0.5	4/4 ≥ 2048	3/3 ≤ 0.5	3/3 ≥ 2048	3/3 ≤ 0.5	2/3 512	3/3 ≤ 0.5	2/3 ≥ 2048	2/3 ≤ 0.5	2/3 ≥ 2048
Control group receiving Hanks BSS treated cells	5/5 ≥ 3.3	5/5 <4	4/4 ≤ 0.5	3/4† ≥ 2048	4/4 ≤ 0.5	4/4 ≥ 2048	4/4 ≤ 0.5	2/4 ≥ 2048	4/4 ≤ 0.5	2/4 512	3/4§ ≤ 0.5	2/4 ≥ 2048

ND = Not Done   Remaining mouse ≥ 2048   Remaining mouse/mice 1024   Remaining mouse 1.0   † Remaining mouse Not done   § Remaining mouse 2.0

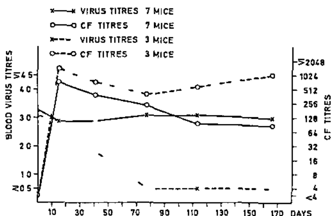


Fig 1

The effect of transplantation of immune ALS treated lymphoid cells to ten syngeneic virus carrier recipients The two alternative courses are shown For further explanation see text

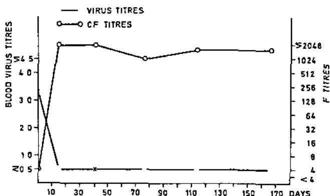


Fig 2

The effect of transplantation of immune NRS treated lymphoid cells to five syngeneic virus carrier recipients

period than in the mice just mentioned (three mice nos 1 4 10) But in these three mice the blood virus content gradually diminished and disappeared within about two months of the transplantation (i e a complete adoptive immunization) Up to the present time the mice have been observed for 166 days and CF and virustitrations are still performed at intervals to discover possible late variations from the course described

The three mice in the experimental group which were completely immunized differed in two respects both from the remainder of the experimental group and from the controls In comparison to the seven partially immunized animals in the experimental group these three mice showed characteristically higher levels of CF antibodies during the whole observation period after transplantation and in comparison

to the controls in which the blood virus was rapidly eliminated these three mice showed a more gradual decrease of the blood virus content over a period of ten weeks

One control group consisted of five female virus carrier mice which were treated with NRS incubated spleen cells from the same donors as those used for the experimental group All five mice were completely adoptively immunized within 15 days after transplantation with virus titres $\leq$ 0 5 and CF titres $\geq$ 2048 All five mice became ill about ten days after transplantation and two died four weeks after transplantation The remaining three recovered slowly but completely and after 166 days observation they were still immune

A second control group also consisted of five female virus carrier mice which were transplanted with Hanks BSS incubated spleen cells from the same donors as those used in the group of experimental animals and the first control group Four of these were also completely immunized within 15 days after transplantation with virus titres $\leq$ 0 5 and CF titres $\geq$ 2048 The fifth died before any virus of CF titrations were performed

Fig 2 shows the NRS treated controls The course of the Hanks BSS treated animals is practically identical to Fig 2 and is therefore not shown in a separate figure

DISCUSSION

If adult LCM virus carrier mice are adoptively immunized by transplantation of 30 $\times$ 10^6 syngeneic LCM immune spleen cells the normal series of events is rapid elimination of virus from the blood associated with very high titres of CF antibodies in the sera in 100 per cent of the animals (16)

The precent experiments however have shown that incubation of the lymphoid cells with ALS prior to transplantation modifies the usual course resulting in a state of partial tolerance (or immunity) characterized by persistent high virus and CF titres in the blood of the animals

Between the 10th and the 30th day after transplantation several animals displayed signs of illness with ruffled pells hypol mesia and conjunctivitis most prominent in the ALS and NRS treated groups This was rather surprising since nothing similar has been observed in hundreds of adoptive immunizations carried out in this laboratory The reason for this might be either a more vigorous immunological conflict in partially immunized animals or else the simultaneous stimulation with other antigens (ALS or NRS)

It was demonstrated that the effect of ALS *in vitro* on LCM immune syngeneic spleen cells was to neutralize their ability (in seven out of ten animals) to eliminate the LCM virus from the blood of tolerant animals The lower CF antibody titre of these animals as compared

with the controls might not be a direct effect of ALS on the humoral antibody formation but could be explained by the persisting high virus level in these animals. This virus could combine with a certain amount of the CF antibodies resulting in smaller measurable titres.

It would thus appear that the effect of ALS on LCM immunity is primarily or exclusively directed against the virus eliminating component of the immune response with little or no effect on the antibody formation component of LCM immunity. Other ALS experiments have demonstrated that ALS acts primarily or exclusively on cell mediated immunities (10, 11) and this therefore adds further support to the accumulating evidence that the virus eliminating function in LCM infection and certain other virus diseases is cell mediated.

Apparently there are at least two different populations of LCM reactive cells in the animals, one which produces humoral antibodies and on which ALS has little or no effect, and one which is responsible for the virus eliminating mechanism and which is sensitive to ALS treatment. It is likely that this latter population represents a thymus dependent cell mediated immunity.

It is noteworthy that the ALS treatment acted so strongly on the presensitized spleen cells that eradication of the cell mediated virus eliminating mechanism was complete in most of the animals. This has also been shown by treatment in vivo of immune animals where a long course of ALS could provoke a new viraemia (17). And it is in line with other findings that ALS may suppress or completely eradicate previously established delayed hypersensitivities to a number of antigens including mumps in animals and man (13).

Nothing certain is known about the way in which the effects of ALS are brought about, but one possibility is that the cells responsible for the cell mediated immunities are coated with ALS and that on injection into the animals with resultant contact with complement they might lyse.

However, in continuation of these experiments it remains to be demonstrated whether the same effect can be obtained by treating either the donors or the recipients in vivo before transplantation. Such experiments are in progress.

In conclusion, it would therefore seem very probable that the immune response which leads to the elimination of LCM virus is brought about by the thymus dependent lymphocytes, while the CF antibodies produced by the gut dependent lymphocytes – only represent a side effect of small or no importance for the pathogenesis. The same state of affairs is thought to govern the immunity to human measles infection (1).

SUMMARY

The effect of ALS on the ability of sensitized lymphoid cells to confer immunity on tolerant virus carrier mice was investigated. LCM im

mune spleen cells were incubated in vitro with ALS and thereafter transplanted into syngeneic LCM tolerant mice These cells were not able to reduce the viraemia in the recipients (7/10 animals) but their ability to produce humoral antibodies was not affected thus leading to a split tolerance in these mice It was argued that the virus eliminating mechanism in LCM immunity (and probably in certain other viral infections) is cell mediated and selectively sensitive to ALS treatment while the production of humoral antibodies (complement fixing) cannot be inhibited Such antibodies are probably of little or no importance in the pathogenesis of LCM disease

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ELECTRON MICROSCOPIC STUDIES ON FLAGELLATION IN DIFFERENT STRAINS OF *YERSINIA ENTEROCOLITICA*

By

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Received 18 iv 69

Bacteria referred to the new species *Yersinia enterocolitica* (*van Loghem* 1944 1946 *Frederiksen* 1964) have been described by various authors (review *Jacober* 1968) as non motile at 37 C but motile at temperatures below 28 C *Frederiksen* (1964) reported the occurrence of peritrichous flagella as a characteristic of the strains studied by him Comparative studies (*Nilén* unpublished observations) of a large number of strains of *Yersinia enterocolitica* of various origins suggested however that non motile or poorly motile variants occur especially often among strains isolated from man dog and pig (O antigen type 3 *Winblad* 1967) as well as among certain biotypes isolated from hare while other hitherto known serological and biochemical types proved to be very motile at temperatures of about 25 C

These observations prompted a comparative investigation of the flagellation of different *Yersinia enterocolitica* strains grown in non defined complete media The *Yersinia enterocolitica* strains were selected in such a way as reasonably to represent different hitherto known serological or biochemical variants This paper is concerned with an electron microscopic examination of the flagellation in strains cultured under uniform conditions at 25 C and with a comparative study of the flagellation in strains cultured at 25 C and 37 C respectively

MATERIAL AND METHODS

Bacterial Strains

The strains used in the study were selected so as to represent the hitherto known antigenic groups (*Winblad* 1967 1968) and different biotypes such as indole positive (ind) strains of mixed origin (*Schleifstein & Coleman* 1939) indole negative

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was then resuspended in sterile saline For electron microscopy negative staining with potassium phosphotungstate (PTA) pH 7 0 and preparation of grids were done according to *Hoeniger's* (1965) modification of the technique of *Thornley & Horne* (1962) Phosphotungstic acid was obtained from Riedel De Haen AG Selze Hannover bovine serum albumin used Protein Standard (I abi AB Stockholm Sweden)

Non formalinized culture Preparations After 3 U tube passages at 25 C bacteria were cultured over night in BEB at 25 C and 37 C respectively A platinum loopful of each broth culture was then sub cultured each loopful in 100 ml of fresh broth The new cultures were maintained at 25 C and 37 C in water baths during continuous shaking For electron microscopy 5 ml samples were taken the cells were washed once with re distilled water and then negatively stained with PTA as described above and used for grid preparations Controls with grid preparations made directly from negatively stained broth cultures were also examined so as to ensure that the flagella were not affected by handling of the cells

The motility of the original over night broth cultures was studied on BEB agar plates at the respective temperatures In some cases cells gently harvested from these plates in sterile re distilled water were examined under the electron microscope

A few poorly motile strains were investigated repeatedly and after different lengths of incubation in order to find motile cells

Investigation of flagellated cultures after temperature shift from 25 C to 37 C U tube passed cells of the strains Bojsen Moller 70 Becht 51 and MY 79b were used in the tests Each strain was inoculated into 100 ml of BEB previously passed through a 1 0 μ membrane filter (Sartorius Membrane Filter Type SM 11003 34/G ttingen Deutschland) The broth culture was incubated over night at 25 C during continuous shaking in a water bath and to a cell density of about 10^8–10^9/ml (viable counts) Half of the culture was then added to 250 ml of fresh BEB 25 C while the other half was transferred to 250 ml of fresh BEB prewarmed to 37 C. The cultures were then incubated during shaking at the respective temperatures At intervals growth was determined by viable counts and measurements of dry weight The pH during growth was continuously registered throughout the observation period of 6 hr For viable counts 0 1 ml samples were collected serially diluted 0 1 ml volumes were spread on human blood agar plates with glass beads and counted after 48 hr at 37 C Five fold plate series from each dilution were counted Dry weigths were determined in 10 ml samples centrifuged at 4 C 2000 g for 45 minutes washed once with re distilled water re centrifuged in the same way re suspended in 0 5 ml of re distilled water and dried for 180 minutes at 80 C.

Bacterial flagella were examined in the electron microscope after negative staining with PTA For staining 1 ml samples of broth culture were mixed with an equal volume of a 2 per cent PTA solution pH 7 0 The approximate number of flagellated cells (per cent) and the average number of flagella per cell (total number of cells) were calculated from counts of at least 200 cells from each preparation

Electron microscopy Formvar and carbon coated copper grids prepared in the way described above were examined in an electron microscope Hitachi HS 70 The proportion of flagellated cells and the number of flagella per cell were determined directly during microscopy The wavelengths (WL) the amplitudes (A) and the lengths of individual flagella were measured from electron micrographs The formula given by *Leifson et al* (1955) was used for calculating the spiral unit lengths (SUL) of the flagella

RESULTS

Flagellation at 25 C

Peritrichous flagella apparently randomly distributed from the bacterial soma could be demonstrated in all of the types of *Yersinia enterocolitica* studied here

Quantitatively however the flagellation differed from one group of strains to another The percentage of flagellated cells in the different strains and maximum number of flagella observed per cell in 48 hr batch cultures at 25 C are given in Table 1 It is clear from the table

(ind.) xylose positive (xyl+) strains of chinchilla or human origin (Mermans & Terpstra 1963, Daniels & Goudzwaard 1963, Nilehn 1967a, b, Nilehn et al. 1968) and xyl strains isolated from man, dog or pig (Hassig et al. 1949, Becht 1962, Dickinson & Mocquot 1961, Nilehn 1967 a, b) and NO_3 non reducing strains (NO_3^-) isolated from hares (Mollaret & Lucas 1965).

Strains Used

Strain no	Origin	O antigen type (Winblad 1967, 1968)	Biotype
1) Becht 51	Chinchilla	1	ind xyl
2) Daniels 924	Chinchilla	2	ind xyl
3) Daniels 1098	Hare	2	NO_3
4) Lucas 404	Hare	2	NO_3
5) Dickinson 07	Pig	3	ind xyl
6) MY 0– Winblad	Man	3	ind xyl
7) MY 2	Man	3	ind xyl
8) MY 57	Man	3	ind xyl
9) MY 60	Man	3	ind xyl
10) MY 134	Man	3	ind xyl
11) Becht Hund 200	Dog	3	ind xyl
12) B Knox 1017/60 61	Chinchilla	4	ind xyl
13) Ye 123–Vache	Cow	5	ind xyl
14) Bojsen Møller 70	Man	6	ind xyl
15) Borg Petersen SP 6613	Guinea pig	7	ind xyl
16) Albany 33114	Man	8	ind xyl
17) Albany 5819	Man	8	ind xyl
18) MY 39	Man	9	ind xyl
19) MY 79b	Man	9	ind xyl

Strains were kindly placed at our disposal by Prof. E. Thal, The State Veterinary Medical Institute, Stockholm (strains 1, 2 and 11 = Thal 348, 338, 356), by Dr. W. Frederiksen, The State Serum Institute, Regional Department, Aalborg (strains 3, 4, 5, 12, 14, 15, 16, 17 = P 372, 368, 253, 76, 213, 413, 310, 311) and by Prof. H. H. Mollaret, Institut Pasteur, Paris (strain 13). Strains 6, 7, 8, 9, 10, 18, 19 from Malmö *Yersinia enterocolitica* collection.

Media

Tryptose glucose broth (TGB). Bacto Tryptose (B 124 Difco) 20 g, glucose 2 g, NaCl 5 g, $Na_2HPO_4 \cdot 2H_2O$ 2.5 g, aq. dest. 1000 ml. Autoclaved for 15 minutes at 120 °C. pH 7.2.

Beef extract broth (BEB). Beef extract (B 126 Difco) 5 g, Proteose peptone no. 3 (B 122 Difco) 10 g, NaCl 3 g, $Na_2HPO_4 \cdot 2H_2O$ 2 g, aq. redest. 1000 ml. Autoclaved for 30 minutes at 120 °C. pH 7.1.

Semi solid agar for motility tests (BEB agar). BEB, Bacto agar (B 140 Difco) 0.5 per cent. Poured into plastic Petri dishes, 9 cm in diameter, 20 ml/dish.

Methods

Strains to be investigated were taken from lyophilized cultures or from stock cultures (+4 °C), in one case (MY 134) a fresh isolate was used.

Formalinized culture preparations. The strains were cultured on human blood agar at 25 °C for 48 hr. After 6 tube passage at 25 °C (BEB agar) cells were inoculated with a platinum loop into a 200 ml volume of TGB in a 500 ml Erlenmeyer retort. The culture was then cautiously shaken for 48 hr at 25 °C in a water bath, after which 0.5 ml of concentrated formalin was added, followed by continued shaking for another 24 hr. Centrifuged at 1060 g for 30 minutes. The deposit

was then resuspended in sterile saline For electron microscopy negative staining with potassium phosphotungstate (PTA) pH 7 0 and preparation of grids were done according to *Hoeniger* s (1965) modification of the technique of *Thornley* & *Horne* (1962) Phosphotungstic acid was obtained from Riedel De Haen AG Selze Hann over bovine serum albumin used Protein Standard (Kabi AB Stockholm Sweden)

Non formalinized culture Preparations After 3 U tube passages at 25 C bacteria were cultured over night in BEB at 25 C and 37 C respectively A platinum loop ful of each broth culture was then subcultured each loopful in 100 ml of fresh broth The new cultures were maintained at 25 C and 37 C in water baths during continuous shaking For electron microscopy 5 ml samples were taken the cells were washed once with re distilled water and then negatively stained with PTA as described above and used for grid preparations Controls with grid preparations made directly from negatively stained broth cultures were also examined so as to ensure that the flagella were not affected by handling of the cells

The motility of the original over night broth cultures was studied on BEB agar plates at the respective temperatures In some cases cells gently harvested from these plates in sterile re distilled water were examined under the electron micro scope

A few poorly motile strains were investigated repeatedly and after different lengths of incubation in order to find motile cells

Investigation of flagellated cultures after temperature shift from 25 C to 37 C U tube passed cells of the strains Bojsen Møller 40 Becht 51 and M 79b were used in the tests Each strain was inoculated into 100 ml of BEB previously passed through a 1 0 μ membrane filter (Sartorius Membrane Filter Type SM 11003 34/Cottingen Deutschland) The broth culture was incubated over night at 25 C during continuous shaking in a water bath and to a cell density of about 10^8–10^9/ml (viable counts) Half of the culture was then added to 250 ml of fresh BEB 25 C while the other half was transferred to 250 ml of fresh BEB prewarmed to 37 C. The cultures were then incubated during shaking at the respective temperatures At intervals growth was determined by viable counts and measurements of dry weight The pH during growth was continuously registered throughout the observation period of 6 hr For viable counts 0 1 ml samples were collected serially diluted 0 1 ml volumes were spread on human blood agar plates with glass beads and counted after 48 hr at 37 C Five fold plate series from each dilution were counted Dry weigths were determined in 10 ml samples centrifuged at 4 C 2000 g for 45 minutes washed once with re distilled water re centrifuged in the same way re suspended in 0 5 ml of re distilled water and dried for 180 minutes at 80 C

Bacterial flagella were examined in the electron microscope after negative stain ing with PTA For staining 1 ml samples of broth culture were mixed with an equal volume of a 2 per cent PTA solution pH 7 0 The approximate number of flagellated cells (per cent) and the average number of flagella per cell (total num ber of cells) were calculated from counts of at least 200 cell from each preparation

Electron microscopy Formvar and carbon coated copper grids prepared in the way described above were examined in an electron microscope Hitachi HS 70 The proportion of flagellated cells and the number of flagella per cell were determined directly during microscopy The wavelengths (WL) the amplitudes (A) and the lengths of individual flagella were measured from electron micrographs The formula given by *Leifson et al* (1955) was used for calculating the spiral unit lengths (SUL) of the flagella

RESULTS

Flagellation at 25 C

Peritrichous flagella apparently randomly distributed from the bacterial soma could be demonstrated in all of the types of *Yersinia enterocolitica* studied here

Quantitatively however the flagellation differed from one group of strains to another The percentage of flagellated cells in the different strains and maximum number of flagella observed per cell in 48 hr batch cultures at 25 C are given in Table 1 It is clear from the table

during the same time of observation Electron microscopy of cells from poorly motile strains harvested from the semi solid agar plates showed the same low proportion of flagellated cells as did investigation of cells from the broth cultures

Flagellation at 37° C

As shown in Table 1 the strains flagellated when incubated at 25 C had practically no flagellated cells when cultured at 37 C under otherwise identical conditions regarding starting material availability and pH of the medium and duration of culture Single flagellated cells observed in two strains had only one single flagellum per cell

To assess the loss of flagella during culture at 37 C 3 very motile type strains representing different serological and/or biochemical variants were selected and studied regarding their flagellation for a 6 hr period after transfer of 25 C—adapted culture to 37 C The effects of this change in temperature are given in Fig 1 a–c where the decrease in the number of flagella per total number of cells respectively the decrease in the percentage of flagellated cells are given in relation to the average number of generations in the culture calculated from log viable counts/ml It is shown in the figures that the sudden temperature shift influenced the bacterial growth in a somewhat different way in the three experiments The strain Becht 51 gave an average generation number of 2 5 during the observation period of 6 hours During this time the number of flagellated cells successively decreased from 85 per cent to about 19 per cent and the mean number of flagella per cell (of the total number of cells) fell from 1 8 to 0 3 As to strain M Y 79b the change in temperature was first followed by a reduction in the number of viable cells by about 50 per cent After a subsequent indefinite lag period the culture yielded on the average 3 1 generations as calculated from the increase of viable counts in the interval between 60 and 360 minutes of the observation period The number of flagellated cells decreased during the same period from about 78 to 19 per cent and the mean number of flagella per cell from 1 6–0 2 Strain Bojsen Moller 70 finally showed a very flat growth curve with on the average only 1 4 generations within 360 minutes The number of flagellated cells decreased during this time from 91 to 66 per cent with a simultaneous decrease of the mean number of flagella per cell from 3 1–1 0 Controls of cultures kept at 25 C but under otherwise identical conditions as those cultured at 37 C invariably showed more than 90 per cent flagellated cells after 6 hours and if anything an increase in the mean number of flagella per cell

TABLE 2

Mean Wavelength (WL), Amplitude (A) and Spiral Unit Length (SUL) of Flagella of Selected Strains of Various Groups of Yersinia enterocolitica. Data in μ. Calculated from Electron Micrographs of Formalinized Cells from Tryptose Glucose Broth Cultures, 48 hr, 25 C. Negatively Stained with Potassium Phosphotungstate

Strain	WL	SD	A	SD	SUL	SD	N
Lucas 404	3.08	–	0.28	–	3.20	–	3
Dickinson 07	2.79	–	0.20	–	2.85	–	2
MY 0	1.24	–	0.14	–	1.47	–	3
MY 134	1.24	–	0.14	–	1.31	–	2
Becht 51	2.83	–	0.31	–	2.99	–	3
Daniels 924	2.78	–	0.27	–	2.90	–	3
B Knox 1017/60–61	2.77	–	0.23	–	2.87	–	2
MY 79b	2.88	0.03	0.33	0.10	3.07	0.69	10
Group mean	2.82	0.05§	0.29	0.04§	2.95	0.03§	
Ye 123 Vache	2.64	0.35	0.25	0.17	2.76	0.42	5
Bojsen Møller 70	2.80	0.17	0.28	0.06	2.94	0.21	10
Borg Petersen 6613	2.83	0.41	0.27	0.08	2.96	0.45	8
Albany 33114	3.05	–	0.31	–	3.20	–	2
Albany 5819	2.80	0.08	0.31	0.04	2.97	0.04	5
Group mean	2.82	0.15§	0.28	0.03§	2.97	0.11§	

§ SD of strain means from group mean
N = number of measurements

TABLE 3

Wavelengths (WL), Amplitudes (A) and Spiral Unit Lengths (SUL) of Flagella in 1 Strain of Yersinia enterocolitica. Data in μ Obtained from Electron Micrographs of Formalin Fixed and Non Fixed Cells from 48 hr Broth Cultures, 25 C

Strain	Preparation		WL	A	SUL
Borg Petersen SP 6613	Formalinized culture		2.96	0.41	3.23
			2.82	0.30	2.97
			2.82	0.26	2.94
			1.94	0.12	1.98
			2.78	0.28	2.92
			2.96	0.26	3.07
			2.90	0.26	3.07
			3.38	0.30	3.51
		Mean	2.83	0.27	2.96
		SD	0.41	0.08	0.4
Borg Petersen SP 6613	Non formalinized culture		3.07	0.32	3.23
			2.71	0.24	2.81
			2.97	0.33	3.14
			2.86	0.26	2.97
			3.02	0.34	3.20
			2.50	0.22	2.59
			2.81	0.21	2.89
		Mean	2.85	0.27	2.98
		SD	0.20	0.05	0.25

that the strains Daniels 1028 and Lucas 404 (isolated from hare) strain Dickinson 07 (from pig) strains MY 0 2 57 60 134 (from man) and strain Becht Hund (from dog) had few or no flagellated cells In the case of these strains examination of the cultures after shorter or longer incubation varying between 6–144 hours revealed no increase in the percentage of flagellated cells One strain (MY 57) gave however about 4 per cent flagellated cells in one of several repeated attempts to segregate motile clones

TABLE 1

Flagellation in Yersinia enterocolitica Strains of Various Types Calculated from Electron Microscopic Counts on Negatively Stained Cells from 48 hr broth cultures at 25 C and 37 C At least 500 Cells of each Strain were Observed

Strain	Biotype	Proportion of flagellated cells (%) 25 C	Proportion of flagellated cells (%) 37 C	Observed maximum number of flagella/cell
Daniels 1028	NO_3	0.1	–	1
Lucas 404	NO_3	0.2	–	3
Dickinson 07	ind xyl	0.3	–	3
MY 0	ind xyl	–	–	–
MY 2	ind xyl	0.1	–	1
MY 57	ind xyl	–(4)	–	–(4)
MY 60	ind xyl	–	–	–
MY 134	ind xyl	0.3	–	2
Becht Hund 200	ind xyl		–	–
Becht 51	ind xyl	70	–	≥ 8
Daniels 924	ind xyl	74	–	≥ 18
B Knox 1017/60 61	ind xyl	39	–	6
MY 39	ind xyl	90	0.2	≥ 10
MY 79b	ind xyl	69	0.4	8
Ye 123 Vache	ind xyl	12	–	7
Bojsen Møller 70	ind xyl	80	–	≥ 18
Borg Petersen SP 6613	ind xyl	89	–	≥ 10
Albany 33114	ind xyl	20	–	6

Figures within brackets denote the flagellation in a culture obtained after repeated attempts to segregate motile clones

As regards ind− xyl+ strains isolated from chinchilla ind− xyl+ strains of human origin (antigen type 9) and ind+ strains of mixed origins the number of flagellated cells varied between 12 and 90 per cent Also the number of flagella per flagellated cell differed in that the cells in poorly flagellated strains had only a single flagellum exceptionally 2–4 flagella per cell while the others were not infrequently richly flagellated but mostly with 2–6 flagella per cell

The motility of the cultures judged from their spreading on a semi solid agar surface varied with the degree of flagellation Strains with high or fairly high percentage of flagellated cells gave growth spreading over the entire surface of the plate in 1–3 days at 25 C while strains belonging in the poorly flagellated group showed no swarming

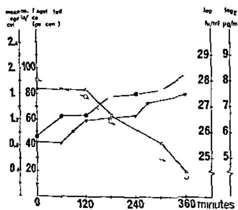


FIG 1a

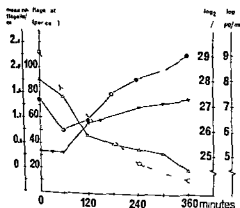


FIG 1b

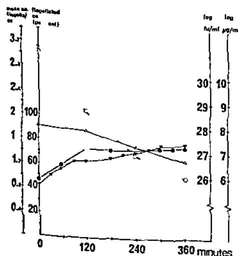


FIG 1c

Fig 1a–c

Decrease in percentage of flagellated cells and mean number of flagella per cell (total cell number) during a 6 hr culture period after temperature shift from 25 C to 37 C in 3 strains of *Yersinia enterocolitica*

●—● log colony forming units (cfu) per ml
▼—▼ log dry weight (µg per ml)
○—○ mean number of flagella per cell (total number of cells)
△—△ percentage of flagellated cells

Fig 1a
Strain Becht 51

Fig 1b
Strain MY 79b

Fig 1c
Strain Bojsen Møller 70

during the same time of observation Electron microscopy of cells from poorly motile strains harvested from the semi solid agar plates showed the same low proportion of flagellated cells as did investigation of cells from the broth cultures

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As shown in Table 1 the strains flagellated when incubated at 25 C had practically no flagellated cells when cultured at 37 C under otherwise identical conditions regarding starting material availability and pH of the medium and duration of culture Single flagellated cells observed in two strains had only one single flagellum per cell

To assess the loss of flagella during culture at 37 C 3 very motile type strains representing different serological and/or biochemical variants were selected and studied regarding their flagellation for a 6 hr period after transfer of 25 C—adapted culture to 37 C The effects of this change in temperature are given in Fig 1 a–c where the decrease in the number of flagella per total number of cells respectively the decrease in the percentage of flagellated cells are given in relation to the average number of generations in the culture calculated from log viable counts/ml It is shown in the figures that the sudden temperature shift influenced the bacterial growth in a somewhat different way in the three experiments The strain Becht 51 gave an average generation number of 2 5 during the observation period of 6 hours During this time the number of flagellated cells successively decreased from 85 per cent to about 19 per cent and the mean number of flagella per cell (of the total number of cells) fell from 1 8 to 0 3 As to strain M Y 79b the change in temperature was first followed by a reduction in the number of viable cells by about 50 per cent After a subsequent indefinite lag period the culture yielded on the average 3 1 generations as calculated from the increase of viable counts in the interval between 60 and 360 minutes of the observation period The number of flagellated cells decreased during the same period from about 78 to 19 per cent and the mean number of flagella per cell from 1 6–0 2 Strain Bojsen Moller 70 finally showed a very flat growth curve with on the average only 1 4 generations within 360 minutes The number of flagellated cells decreased during this time from 91 to 66 per cent with a simultaneous decrease of the mean number of flagella per cell from 3 1–1 0 Controls of cultures kept at 25 C but under otherwise identical conditions as those cultured at 37 C invariably showed more than 90 per cent flagellated cells after 6 hours and if anything an increase in the mean number of flagella per cell

TABLE 2

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Strain	WL	SD	A	SD	SUL	SD	N
Lucas 404	3 03	–	0 8	–	3 ?0	–	3
Dickinson 07	2 79	–	0 ?0	–	2 85	–	2
MY 0	1 24	–	0 14	–	1 47	–	3
MY 131	1 24	–	0 14	–	1 31	–	?
Becht 51	? 83	–	0 31	–	? 99	–	3
Daniels 9?4	? 78	–	0 ?7	–	? 90	–	3
B Knox 1017/C0 61	? 77	–	0 ?3	–	2 87	–	2
MY 79b	2 88	0 63	0 33	0 10	3 07	0 69	10
Group mean	2 8?	0 05§	0 29	0 04§	2 9?	0 09§	
Ye 123 Vache	? 64	0 35	0 2?	0 17	2 76	0 4?	5
Bojsen Møller 70	2 80	0 17	0 ?8	0 06	? 94	0 21	10
Borg Petersen 6613	? 83	0 41	0 27	0 08	? ?6	0 45	8
Albany 33114	3 05	–	0 31	–	3 20	–	2
Albany 5819	2 80	0 08	0 31	0 04	? 97	0 04	5
Group mean	2 82	0 15§	0 ?8	0 03§	2 97	0 1?§	

§ SD of strain means from group mean
N = number of measurements

TABLE 3

Wavelengths (WL) Amplitudes (A) and Spiral Unit Lengths (SUL) of Flagella in 1 Strain of Yersinia enterocolitica Data in μ Obtained from Electron Micrographs of Formalin Fixed and Non Fixed Cells from ?8 hr Broth Cultures 2? C

Strain	Preparation	WL	A	SUL
Borg Petersen SP 6613	Formalinized culture	? 96	0 41	3 23
		? 8?	0 30	2 97
		2 82	0 ?6	2 94
		1 94	0 1?	1 98
		? 78	0 ?8	? 9?
		? 9?	0 ?6	3 07
		2 9?	0 ?	3 07
		3 38	0 30	3 51
	Mean	? 83	0 ?7	? 9?
	SD	0 41	0 08	0 45
Borg Petersen SP 6613	Non formalinized culture	3 07	0 3?	3 ?3
		2 71	0 ?4	? 81
		2 97	0 33	3 14
		2 8?	0 ?6	? 97
		3 0	0 34	3 0
		2 50	0 ??	? 59
		? 81	0 21	2 89
	Mean	? 8	0 ?7	2 98
	SD	0 ?0	0 05	0 2?

during the same time of observation Electron microscopy of cells from poorly motile strains harvested from the semi solid agar plates showed the same low proportion of flagellated cells as did investigation of cells from the broth cultures

Flagellation at 37 C

As shown in Table 1 the strains flagellated when incubated at 25 C had practically no flagellated cells when cultured at 37 C under otherwise identical conditions regarding starting material availability and pH of the medium and duration of culture Single flagellated cells observed in two strains had only one single flagellum per cell

To assess the loss of flagella during culture at 37 C 3 very motile type strains representing different serological and/or biochemical variants were selected and studied regarding their flagellation for a 6 hr period after transfer of 25 C—adapted culture to 37 C The effects of this change in temperature are given in Fig 1 a–c where the decrease in the number of flagella per total number of cells respectively the decrease in the percentage of flagellated cells are given in relation to the average number of generations in the culture calculated from log viable counts/ml It is shown in the figures that the sudden temperature shift influenced the bacterial growth in a somewhat different way in the three experiments The strain Becht 51 gave an average generation number of 2 5 during the observation period of 6 hours During this time the number of flagellated cells successively decreased from 85 per cent to about 19 per cent and the mean number of flagella per cell (of the total number of cells) fell from 1 8 to 0 3 As to strain MY 79b the change in temperature was first followed by a reduction in the number of viable cells by about 50 per cent After a subsequent indefinite lag period the culture yielded on the average 3 1 generations as calculated from the increase of viable counts in the interval between 60 and 360 minutes of the observation period The number of flagellated cells decreased during the same period from about 78 to 19 per cent and the mean number of flagella per cell from 1 6–0 2 Strain Bojsen Møller 70 finally showed a very flat growth curve with on the average only 1 4 generations within 360 minutes The number of flagellated cells decreased during this time from 91 to 66 per cent with a simultaneous decrease of the mean number of flagella per cell from 3 1–1 0 Controls of cultures kept at 25 C but under otherwise identical conditions as those cultured at 37 C invariably showed more than 90 per cent flagellated cells after 6 hours and if anything an increase in the mean number of flagella per cell

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Strain	WL	SD	A	SD	SUL	SD	N
Lucas 404	3.08	–	0.28	–	3.20	–	3
Dickinson 07	2.79	–	0.20	–	2.85	–	2
MY 0	1.24	–	0.14	–	1.47	–	3
MY 134	1.24	–	0.14	–	1.31	–	2
Becht 51	2.83	–	0.31	–	2.99	–	3
Daniels 924	2.78	–	0.27	–	2.90	–	3
B Knox 1017/60–61	2.77	–	0.23	–	2.87	–	2
MY 79b	2.88	0.63	0.33	0.10	3.07	0.69	10
Group mean	2.82	0.05§	0.29	0.04§	2.9	0.09§	
Ye 123 Vache	2.64	0.35	0.25	0.17	2.76	0.42	5
Pojsen Moller 70	2.80	0.17	0.28	0.06	2.94	0.21	10
Borg Petersen 6613	2.83	0.41	0.27	0.08	2.96	0.45	8
Albany 33114	3.05	–	0.31	–	3.20	–	2
Albany 5819	2.80	0.08	0.31	0.04	2.97	0.04	5
Group mean	2.82	0.15§	0.28	0.03§	2.97	0.16	

§ SD of strain means from group mean
N = number of measurements

TABLE 3

Wavelengths (WL), Amplitudes (A) and Spiral Unit Lengths (SUL) of Flagella in 1 Strain of Yersinia enterocolitica *Data in μ Obtained from Electron Micrographs of Formalin Fixed and Non Fixed Cells from 48 hr Broth Cultures 25 C*

Strain	Preparation		WL	A	SUL
Borg Petersen SP 6613	Formalinized culture		2.96	0.41	3.23
			2.82	0.30	2.97
			2.82	0.26	2.94
			1.94	0.12	1.98
			2.78	0.28	2.92
			2.96	0.26	3.07
			96	0.26	3.07
			3.38	0.30	3.51
		Mean	2.83	0.27	2.96
		SD	0.41	0.08	0.45
Borg Petersen SP 6613	Non formalinized culture		3.07	0.32	3.23
			2.71	0.24	2.81
			2.97	0.33	3.14
			86	0.26	2.97
			3.02	0.34	3.20
			2.50	0.22	2.59
			2.81	0.21	2.89
		Mean	2.85	0.27	2.98
		SD	0.20	0.0	0.25

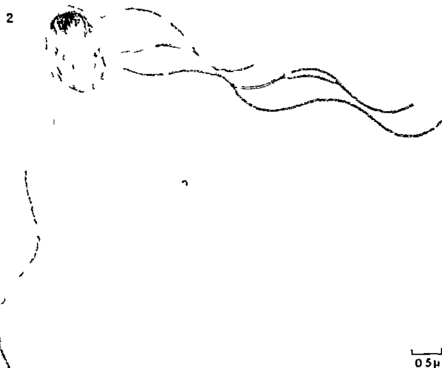


Fig 2

Strain Bojsen Moller 70 and O antigen type 6 human origin Flagella demonstrated after 48 hours incubation in tryptose glucose broth at 25 C Formalin fixation negative staining with PTA

Flagellar Morphology

Some data on the morphology of the flagella of the various strains after formalin fixation are given in Table 2 where wavelengths (WL) amplitudes (A) and spiral unit lengths (SUL) (*Leifson et al* 1955) of different strains have been recorded As is apparent from the table and also shown in Figs 2–7 relatively wide variations in the wave lengths and amplitudes were seen not only within different strains but also often in the case of flagella of one and the same bacterial cell (Fig 4) Flagella with a double curvature (*Leifson* 1960) were also observed (Fig 6) and in some cases straight flagella were seen (Fig 3) Maximum flagellar length in the formalinized preparations of the different strains varied between 7 and 11 6 μ

Formalin fixation did not decrease the variation of the wavelength or of the amplitude in individual strains as compared with the result of examination of a non fixed culture (Table 3)

Among strains investigated after formalin fixation the strains M Y 134 and M Y 0 showed the average shortest mean wavelength (1 24 μ) with a mean amplitude of 0 14 μ the number of measurements was however very small owing to the sparsity of the flagella Measure



Fig 3

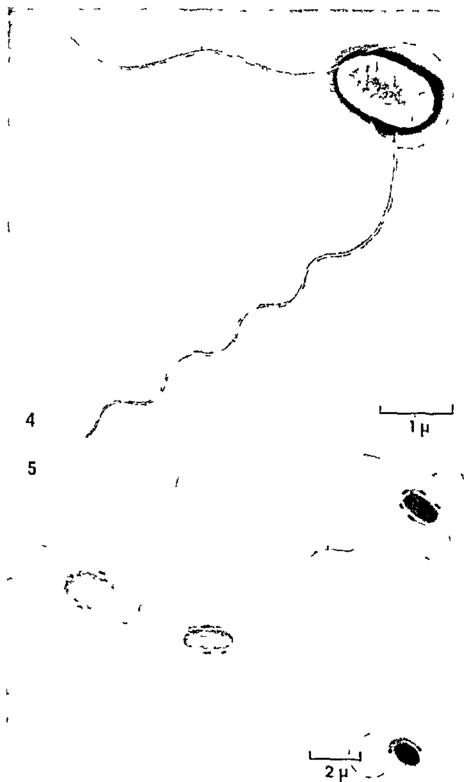
Strain MY 39 and xyl O antigen type 9 human origin Cells abundantly flagellated and showing several straight flagella Incubation 48 hr in beef extract broth at 25 C Non fixed preparation negative staining with PTA

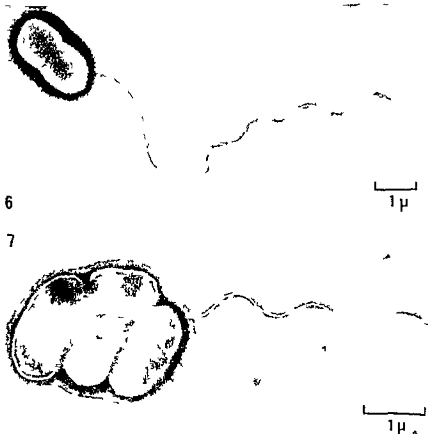
ments of non fixed preparations of strain MY 57 belonging to the same antigen type 3 gave wavelength means of 1 32 µ (SD 0 14) and 1 24 µ (SD 0 09) after incubation for 48 hours and 96 hours respectively

DISCUSSION

The purpose of the present investigation was twofold *viz* 1) to find out whether divergencies in flagellation or flagellar morphology occur in parallel with differences in motility among strains referred to *Yersinia enterocolitica* and 2) whether such divergencies if any may also be responsible for the variation in the motility of motile strains with temperature

The results did not indicate any differences between various groups of strains with regard to the arrangement of the flagella in agreement with previous observations (*Frederiksen* 1964) peritrichous flagella could be demonstrated in all groups studied Flagellation however was found to differ quantitatively in the different strains The two strains isolated from hare and and− xyl− strains isolated from man dog and pig showed no flagella or very few flagella compared with the other well flagellated groups Poorly motile human strains as judged from the few possible measurements also showed a shorter wavelength than did strains that were richly flagellated The quanti


4
1 µ
5
2 µ



Figs 6–7

Fig 6 *Strain* MY 57 and xyl O antigen type 3 human origin Single flagellum with double curvature Incubation for 24 hr in beef extract broth at 25 C Non fixed preparation negative staining with PTA

Fig 7 *Strain* MY 134 and xyl O antigen type 3 human orig n Flagellated cell in formalinized preparation of 48 hr culture in tryptose glucose broth 25 C Negative staining with PTA

tative differences in flagellation were well correlated with the swarming ability of the bacteria on the surface of semi solid agar The results thus suggest that the weak locomotor activity of poorly motile strains is due to lack of flagella rather than to the occurrence of abnormal flagella or to flagellar paralysis

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Figs 4–5

Strain Daniels 924 and xyl O antigen type 2 chinchilla origin Flagellated cells harvested from semi solid agar plates 48 hr incubation 25 C showing flagella of different wavelength and amplitude Non fixed preparation negative staining with PTA

A paucity or absence of flagella may be a result of development of non flagellated cells in a certain laboratory environment or of development of non flagellated mutants The influence of different environmental factors such as composition of medium pH age of the culture and temperature on the synthesis and persistance of flagella of different bacteria has long been known (*Oguti* 1936 *Weibull & Tiselius* 1945 *Leifson et al* 1955 *Leifson* 1960 *Stocker & Campbell* 1959 *Quadling & Stocker* 1962 *Gerber & Noguchi* 1967) Essential precursors and a suitable source of energy for the synthesis of flagella in a synthetic medium have been studied in *E coli* by *Adler & Templeton* (1967) These authors also demonstrated that the motility was inhibited by heavy metal ions and glucose Also *Leifson* (1960) reported the inhibitory effect of fermentable carbohydrates on the synthesis of flagella

The strains under discussion have been examined in non defined complete media buffered to pH 7 2–7 0 which have invariably given good growth at both 37 C and 25 C It is not likely that there was any deficiency of precursors necessary for the synthesis of flagella It seems more reasonable to assume that medium components present or formed as intermediate metabolites may inhibit the synthesis of flagella of certain biotypes Preliminary experiments have thus shown that EDTA in concentrations between 10^{-4} and 10^{-6} M enhances the motility of strains in the poorly motile group (*Alehn* 1968 unpublished observations) similar observations concerning *E coli* have been reported previously by *Adler & Templeton* (1967)

Poorly flagellated strains examined in cultures of different ages did not show an increased number of flagella in the earlier phases of growth Neither were there any differences between a newly isolated strain and older stock cultures or lyophilized strains

The effect of the temperature on the motility of different *Yersinia enterocolitica* strains has been observed by several investigators (for review see *Jacober* 1968) The present results indicate that the non motility at 37 C of strains motile at 25 C is due to a loss of flagella when cultured at the higher temperature The transfer of cultures from 25 C to 37 C did not result in a complete loss of flagella immediately after the change in temperature but in a successive decrease An exact determination of this decrease as a function of the rate of bacterial growth is possible only if the initial proportion of viable cells of the culture and the number of viable cells per colony forming unit are known The effect of the sudden change in temperature on viability continued cell functions and existing flagella in the different tested cultures is also difficult to assess the initial viable counts after transfer to 37 C differed however only in one case (strain MY 79b) from those of the control culture at 25 C The culture reacted in this case with persisting or transient loss of viability in more than half of the cells But though the results do not allow any detailed analysis of the loss of flagella in relation to cell division they do

suggest a cessation of flagellar synthesis after transfer to the culture to 37 C while synthesis of flagella continued in the control cultures kept at 25 C

Variations in the metabolic activity with temperature have been found for this group of bacteria *e g* regarding catabolism of certain carbohydrates (*Niléhn* 1967 a b) or requirements of different growth factors (*Burrows & Gillett* 1966) The mechanism of the possible blocking of synthesis of flagella at the higher temperature is however obscure and has as far as is known not been studied

The genetic stability of flagella encountered in single cells in the poorly flagellated group was not studied In one case after repeated attempts to find motile clones one culture yielded about 4 per cent flagellated cells With the aid of micromethods it might be possible under the experimental conditions used to isolate cells yielding a progeny with stable flagellation

The flagellar shape varied widely in individual strains an observation also applying to the closely related *Yersinia (Pasteurella) pseudotuberculosis* (*Leifson* 1960) Variations in the form of biplicity double curvature or straight flagella were observed both in formalin fixed and in non fixed preparations Whether the differences in mean wave length between certain poorly motile strains of human origin and other groups were significant cannot be decided until a larger number of strains have been studied

It is difficult to assess the possible taxonomic significance of the quantitative and qualitative differences in flagellation in different groups of strains until more knowledge has been obtained of the genetic or physiological background In this conjunction it should however be pointed out that poorly flagellated strains isolated from man dog and pig resemble one another also in biochemical properties (*Nilehn* 1967 b) antigenic components (*Winblad* 1967) and phage sensitivity (*Nicolle et al* 1967 *Nilehn & Ericson* 1969) As an entity the *Yersinia enterocolitica* group seems as regards its flagellation to occupy an intermediate position where the flagellated variants resemble the otherwise closely related *Yersinia pseudotuberculosis* while strains poor in flagella and above all the biochemically peculiar group of hare origin approach the biochemically related non motile *Yersinia (Pasteurella)* pestis

SUMMARY

Electron microscopic investigation of the flagellation of *Yersinia enterocolitica* strains representing various serological and/or biochemical variants showed the occurrence of peritrichous flagella at 25 C in all groups examined

On examination of the strains in cultures in non defined complete media they differed quantitatively in their flagellation There was thus one richly flagellated motile group and one very scantily flagellated

poorly motile group the latter comprising strains belonging to certain biotypes isolated from man dog pig or hare

Richly flagellated strains were often characterized by a relatively large variability in wavelength and amplitude and also in the shape of individual flagella or flagella of single cells

A successive loss of flagella of 3 type strains was observed after transfer of 25 C—adapted cultures to 37 C

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BIOSYNTHETIC STABILITY OF THE TOXIGENETIC CAPACITY OF *CLOSTRIDIUM TETANI* ON REPEATED TRANSFER IN CULTURE MEDIA

By

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Received 28 V 69

It has often been observed during the preparation of tetanus toxin (*Mueller & Miller* (10) *van Hemert* (6) *Scheibel* (18)) that highly toxigenic *Cl tetani* loose their toxin producing ability to varying extent on repeated subculture

It has been reported recently that repeated transfers of a strain of *Cl perfringens* also resulted in a decrease of the toxigenicity (*Soda et al* (19))

Such instability in biosynthesis is also known from other micro biological fields e g in antibiotic production (*Reusser* (17))

Very little is known concerning the mechanism involved and the factors which cause the decrease in or loss of the toxin producing ability

In order to obtain a highly yield of toxin with the *Cl tetani* strain in question it is necessary to utilize a specific medium (*Mueller & Miller* (9 11 12 13) *Fisek et al* (5) *Latham et al* (7))

As is reported in the above mentioned literature and furthermore by *Mueller & Miller* (8) *Mueller et al* (14) and *Feeney et al* (2 3) the strain has rather complex nutritional requirements However it does not demand such specific conditions for growth as for toxin production since it grows well in different kinds of media without producing more than traces of toxin

It has been observed (*Nielsen* (15)) that the above mentioned de crease in toxigenicity is dependent to some degree on the medium used for subculture This dependency is being investigated and some pre liminary results are given in the present paper

MATERIAL AND METHODS

Medium I (special peptone meat infusion)

Peptone meat infusion medium with 0 25 per cent w/v of thioglycollic acid and 5 per cent w/v of Fildes peptic blood broth (horse blood) is made according to the following technique

0 5 kg minced beef muscle is mixed with 0 5 litre tap water stored at + 5 C

overnight and then boiled for 10 minutes and strained through cloth Tap water is added to 1 0 litre and the infusion is boiled for 10 minutes 10 g peptone (Orthana Special Peptone) 3 g sodium chloride and 2 g sec sodium phosphate (with 12 H O) are then added This mixture is boiled for 5 minutes and the pH is adjusted to 7 5 with sodium hydroxide Filtration is made through paper and then through Seitz filter The volume is adjusted to 1 0 litre with distilled water and the mixture heated to 100 C for 10 minutes The pH should be 7 4 To this infusion is added 0 05 per cent w/v thioglycollic acid and 5 per cent v/v Fildes peptic blood broth Fildes peptic blood broth is made from horse blood with some minor modification of the original prescription (*Fildes* (4)) the digestion being carried out at 56 C for six hours only The final medium is filled into test tubes or containers and autoclaved

Medium I is occasionall used as a semi solid product obtained by the addition of 0 2 per cent w/v agar

The Cl tetani strain in question grows rapidly and copiously in this medium but produces only traces of toxin

Medium II (current medium for toxin production)

This is a slightly modified form of the medium described by *Mueller & Miller* (11) A commercial product Tryptone Oxoid i used instead of pancreatic digest of casein or N Z Case and soluble ferrous sulphate is used instead of reduced iron The composition of the medium is shown in Table 1

TABLE 1

Composition of Medium II which is a slight Modification of the Mueller & Miller Medium for Tetanus Toxin Production

Components	*per litre*	
Tryptone Oxoid	25 0 g	
Ox heart extract	50 0 ml	equivalent to 45 8 g of fresh ox heart
Glucose	11 0 g	
NaCl	2 5 g	
Na HPO	2 0 g	
KH PO	0 15 g	
MgSO 7H O	0 15 g	
l cystine	0 25 g	
l tyrosine	0 5 g	
Ca pantothenate	1 0 mg	
Uracil	2 5 mg	
Thiamine	0 25 mg	
Riboflavine	0 5 mg	
Pyridoxine	0 25 mg	
Biot n	5 mcg	
FeSO 7H O	40 0 mg	
D stilled water	to 1 0 litre	

This medium in which the *Cl tetani* strain in question both grows well and produces high concentrat on of toxin has been us d for many years in the routine production of tetanus toxin at Staten Se uminstitut Copenhagen

It has been observed that the yield of tox n may vary with the batch of Tryptone Oxoid used Ho e er prelim nary *tr atment of the tryptone with charcoal* eliminated most of th e variat ons The optimum effect of charcoal treatment is obtained by adding 7 g charcoal (Norit) per litre of a 10 per cent Tryptone Oxoid solution in dist lled water

In some experiments medium II with an add tion of 0 25 per cent w/v thioglycollic acid was used in rder to obtain a lower redox potential

Med um III (semi solid g r bou llon with glucose)

Meat infusion broth with 1 pe c nt w/v peptone (Orthana special) 0 3 per c nt

w/v NaCl 0 2 per cent w/v Na HPO$_4$ 12H O 0 2 per cent w/v glucose and 0 2 per cent w/v agar

Strain

The non sporulating strain of *Cl tetani* used in this work has been used for routine production of tetanus toxin at Statens Seruminstitut Copenhagen since 1951 and also in many other countries

The strain originated from a freely sporulating strain collection No 298 which Dr Howard Mueller Harvard Medical School Boston received from the Division of Laboratories on Research New York State Health Department Albany N Y in 1939

In Dr Mueller s laboratory the strain no longer sporulated freely after daily subculture for some months in peptone meat infusion broth with 1 per cent glu cose in the presence of CO and H

A culture of the non sporulating variant No 43415 was returned to Albany in 1943 from which source it was received in this laboratory in 1949

In our laboratory the strain is kept as a lyophilized culture made from a two day transfer in medium III

Cultivation Methods

1) *Subculture* The above mentioned media were used for subculture as de scribed under each experiment and cultivation was performed at 34 C in test tubes (155 mm high internal diameter 12 mm) containing 10 ml medium The amount of inoculum was 0 2 ml The cultivation time is stated under each ex periment this was usually from 1 to 3 days Each transfer was controlled for purity on blood agar dishes both aerobically and anaerobically

2) *Toxigenicity testing* (toxin production) The ability to produce tetanus toxin was investigated by cultivation in test tubes under the same conditions as those mentioned above except that the growth period was prolonged to 10 days Fil tration through ordinary filter paper was then carried out toluene was added to the filtrate and toxin titration was performed Toxigenicity testing was carried out either in medium II (without thioglycollic acid) or in medium I with and without agar

Titration of Toxin in vitro

1) *Flocculation (Lf)* The test was carried out by the Ramon or Lf method *i e* varying amounts of a local reference antitoxin (0 1–1 0 ml with a dose interval of 0 10 log) were mixed with a constant amount (1 0 ml) of the toxin to be tested The tubes were placed in waterbath at 48 C until floculation had taken place The titre is calculated from the first tube showing flocculation and expressed in Lf/ml The limit of error under these conditions is about ± 10 per cent of the observed result

2) *Mixed floculation* The toxin to be tested was mixed in a ratio of 1 1 with a local reference toxin known to give only specific flocculation with the anti toxin used Other conditions were as described under point 1

The mixed flocculation technique prevents to a certain degree *misreading* caused by false flocculation zones In spite of the much wider limit of error especially with low toxin values (for Lf about 3–6 ± 100 per cent for Lf about 10 ± 62 per cent of the observed value) as compared to those of the direct Ramon test this method is useful for estimation of the Lf titre in toxins of low value *i e* below 10 Lf per ml with which it is difficult to obtain flocculation by the direct method

The mixed flocculation technique was used as supplementary test in all cases where there was reason to doubt the specificity of the direct flocculation and where more than one floculation zone occurred Thus the Lf titres given in the curves indicate with reasonable certainty the specific antitoxin combining ca pacity in the various filtrates In most of the experiments this has been verified by in vivo titrations

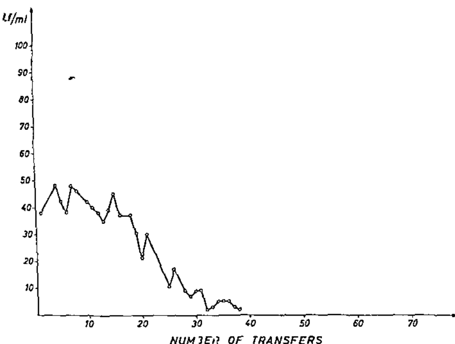


Fig 1

Derease in toxigenicity by subculture of Cl tetani strain 43415 in medium II using 38 transfers over a period of 76 days
Open circles represent the Lf per ml in a 10 day old culture in medium II of the corresponding transfer
Abscissa Number of transfers
Ordinate Lf/ml

Titration of toxin in vivo

Antitoxin neutralisation (L) The test was carried out in white mice (weight 16–18 g) by injecting mixture of one tenth of an international antitoxin unit and varying amounts of the toxin to be tested the endpoint of the titration being the mixture that killed 50 per cent of the animals in five days

Generally dose intervals of 0 10 log were used and t v mice v ere injected per dose Statistical evaluation of a series of titrat on in our laboratory has dis closed that the limit of error under these conditions was ± 1? per cent of the observed value

The L titre of tetanus toxin is generally exp es ed n L /10/50 per ml being as sayed against one tenth of an antitoxin unit How v r to facilitate comparison with the Lf titre which is based on a level of one antitoxin unit the number of L /10/50 per ml found by the in vivo titration i divided by 10 and shown as L /1/50 in the present work

RESULTS

Successive subculture of *Cl tetani* strain 13415 in medium II led to a systematic loss in toxigenicity Fig 1 shows the result of such an

Thanks are due to Mr Weis Bent on Biostatistical Department for making the statistical evaluations

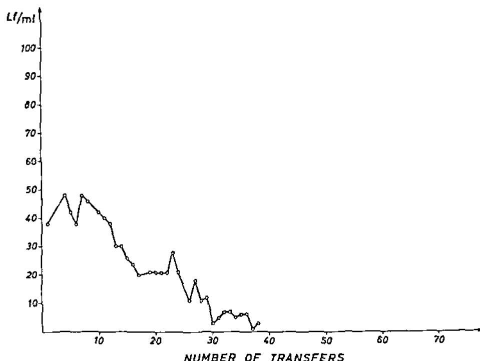


Fig 2

Decrease in toxigenicity by subculture of Cl tetani strain 43415 in medium II containing 0 25 per cent w/v thioglycollic acid Open circles represent the Lf per ml in a 10 day old culture of the corresponding transfer The first transfers are the same as in Fig 1 From transfer No 13 thioglycollic acid was added to the medium The 38 transfers cover a period of 76 days
Abscissa Number of transfers
Ordinate Lf/ml

experiment where 38 transfers were made over a period of 76 days (3 times weekly)

The toxigenicity of the subculture was tested in medium II as described under toxigenicity testing The open circles represent the toxin production of the strain expressed in Lf/ml It will be seen that after about 15 transfers a marked decrease in Lf occurred and that after about 30 transfers only very low values were obtained

The Lf titres at the beginning and end of this and the next experiment were checked by in vivo titration and the two parameters showed a reasonable degree of agreement

Fig 2 shows the results of a similar experiment except that after transfer No 13 0 25 w/v per cent thioglycollic acid was added to the medium The resulting lower redox condition did not seem to influence essentially the loss in toxigenicity during the subsequent subcultures

As the decrease in toxigenicity progressed unspecific flocculation zones occurred with increasing frequency

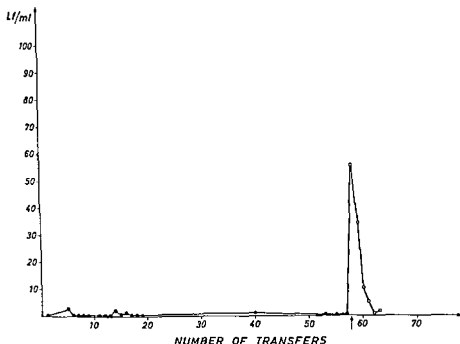


Fig 3

Influence on the toxigenicity of Cl tetani strain 43415 of 57 transfers in medium I with agar and 5 additional transfers in medium II
The dots show the toxin production in Lf per ml in a 10 day old culture in medium I of the corresponding transfer of the Cl tetani strain 43415 in medium I with agar
The open circles indicate the toxin production in a 10 day old culture in medium II of the corresponding subculture
Abscissa Number of transfers
Ordinate Lf/ml

As reported previously (15) the decrease in toxigenicity of *Cl tetani* strain 43415 by repeated subculture in semi solid agar bouillon with glucose (medium III) was much less marked than when subcultured in medium II

When subculture was carried out in medium I in which as mentioned the strain grows exceptionally fast and gives copious growth but produces only minute amounts of toxin the toxigenicity appeared to be stable through a considerable number of transfers

In the present work the number of consecutive subcultures in medium I was extended to more than 100 over a period of more than 30 weeks without any demonstrable decrease in toxigenicity as judged by 10 day old cultures in medium II

However some change in the metabolic properties of the strain seems to have taken place since after a number of transfers in medium I the toxigenicity decreased at an accelerated rate on subsequent subculture in medium II A typical experiment to demonstrate this is

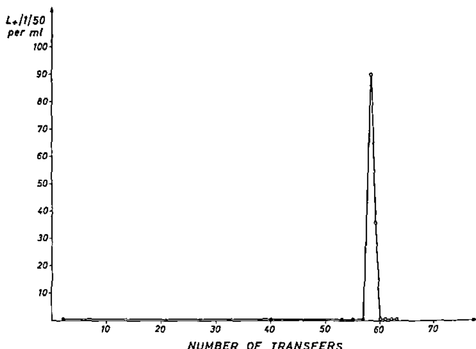


Fig 4

In vivo titration of the same culture filtrates as shown in Fig 3 The toxin production is given in L+/1/50 units per ml to enable a comparison with the Lf titres in Fig 3
Abscissa Number of transfers
Ordinate L+/1/50 per ml

shown in Fig 3 The strain was transferred 57 times in medium I with 0 2 per cent of agar the transfers being carried out every second day over a period of 17 weeks

The dots in the diagram show the toxin production in Lf per ml obtained after 10 days growth of the corresponding transfers in medium I without agar As can be seen the strain produced only very little toxin in this medium over the whole period

After transfer No 57 the strain was transferred to medium II in which five additional transfers were performed The toxin production in Lf per ml in a 10 day old culture in medium II of the corresponding transfers is indicated by the open circles

Transfer No 57 gave a high yield of toxin in medium II but after only one subculture in this medium there was a decrease in toxin production from 56 to 34 Lf/ml After three to four transfers in medium II the toxin production had decreased to a very low level As can be seen from Figs 1 and 2 it took more than 25 transfers to invalidate the toxigenicity to the same degree without previous subculture in medium I

In this experiment in vivo titrations of each batch were also carried

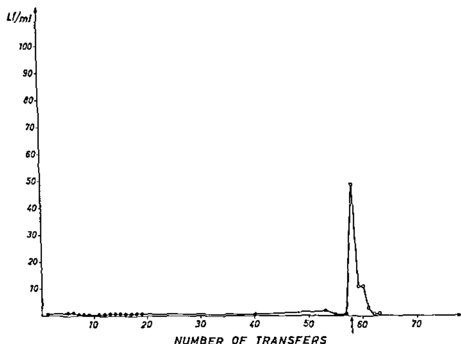


Fig 5
Influence on the toxigenicity of Cl tetani 43415 of 57 transfers in medium I without agar on additional transfers in medium II
The dots show the toxin production in medium I without agar and the open circles the toxin production in medium II of the corresponding subculture
Abscissa Number of transfers
Ordinate Lf/ml

out the results are shown in Fig 4 During the first 10 days culture in medium II the L + titre was significantly higher than the Lf titre whereas that was not the case for the later cultures in this medium However on the whole the antitoxin combining capacity as measured in vivo confirms the result found by Lf titration

Figs 5 and 6 show the results of a similar experiment in which the first 57 transfers were made in medium I without the addition of agar Both the in vivo and the in vivo titration gave results similar to those found in the experiment presented in Figs 3 and 4 Again the L+ titre of the first 10 day old culture in medium II was considerably higher than the Lf titre

It was then investigated whether the low toxigenicity induced by repeated transfers in medium II could be regenerated by subculture in a medium which stabilizes the toxigenicity

For this purpose a medium II culture which was reduced from a toxin producing ability of more than 50 Lf/ml to practically no toxigenicity viz No 46 of a series of successive transfers was subcultured

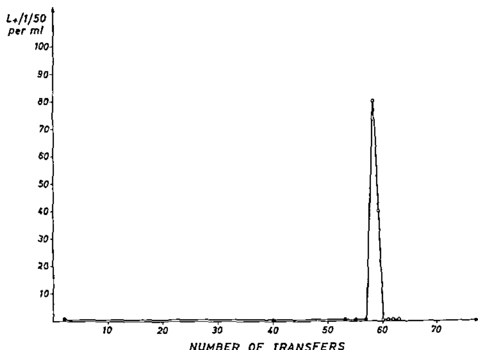


Fig 6

In vivo titration of the same culture filtrates as shown in Fig 5 The toxin pro duction is given in L /1/50 units per ml to enable comparison with the Lf titres in Fig 5
Abscissa Number of transfers
Ordinate L /1/50 per ml

three times in medium I with and without addition of agar and in medium III

The third transfer in each medium was then tested for toxin production in medium II in the ordinary way

The results are presented diagrammatically in Fig 7 Only the L+/1/50 titres are given the Lf values as mentioned being difficult to measure with a satisfactory degree of certainty at these low levels

The results for medium I with agar medium I without agar and medium III are shown in Curves A B and C respectively

It was ascertained by toxigenicity testing of the three transfers in each of the three different media that no toxin production which could be measured by the method used took place in any of the transfers On cultivation of the third transfer in medium II some but only very little toxin was produced in comparison with that obtained when the strain was subcultured only in medium I (cf Figs 3 4 5 and 6) The same applied to all three media tested

On further subculture in medium II the acquired slight toxigenicity was again lost after only one transfer

In the next experiment a culture of the lyophilized stock of *Cl tetani*

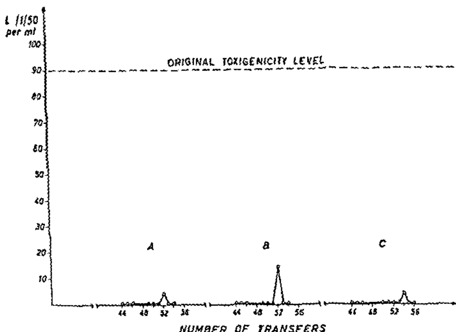


Fig 7

Influence of three successive transfers in medium I with agar medium I without agar and medium III on the toxigenicity of Cl tetani strain 43415 which has lost its toxin producing ability during 46 transfers in medium II
Toxigenicity determined by renewed subculture in medium II
Open circles show the toxin production in medium II dots the toxin production in medium I with agar closed triangles the toxin production in medium I without agar and open triangles the toxin production in medium III all of the corresponding transfers number
Abscissa Number of transfers
Ordinate L /1/50 per ml

No 43415 used for routine production which had been transferred nine times partly in medium II and partly in medium III and thereby lost about 70 per cent of its original toxigenicity was transferred to medium I with agar and subcultured in this medium during a period of about two years

219 subcultures in medium I (total number of transfers 228) were made at varying intervals and the toxin production in medium II was investigated on a number of these the aim being to ascertain whether prolonged series of transfers in medium I would reestablish an only partly lost toxigenicity

The results is presented in Fig 8 the L + /1/50 titre being the parameter for the toxin production

The titre of the original 10 day-old lyophilized culture was 50 L + /1/50 per ml and that of the ninth subculture in medium II and medium III 15 None of the first 46 transfers in medium I tested for

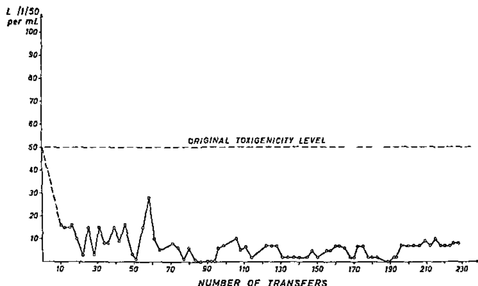


Fig 8

Toxigenicity during 228 transfers of Cl tetani strain 43415 carried out over a period of two years The first nine transfers were made in medium II and medium III by which procedure the strain lost about 70 per cent of its toxigenicity The following 219 transfers were carried out in medium I with agar The toxigenicity indicated by open circles was tested by inoculation in medium II and growth for 10 days
Abscissa Number of transfers
Ordinate L /1/50 per ml

toxigenicity in medium II exceed that latter level but showed fluctuations around a mean of 10 L+/1/50

Transfer No 49 in medium I (total number of transfers 58) caused an increase in titre to about 30 L+/1/50 per ml after which the titres varied between 0 and about 10 for the rest of the period On the whole there was no indication of a regeneration of the original toxin producing capacity

DISCUSSION

The highly toxigenic strain No 43415 used in the present investigation is probably an asporogenic mutant of the commonly found low toxigenic strains To the writer s knowledge this strain and variants of it are the only highly toxigenic tetanus strain reported hitherto The type of genetic change causing the high toxigenicity is not known It is tempting to assume a bacteriophage as the influencing agent but up to now no report has been made of any bacteriophage connection with the toxigenicity of *Cl tetani* strains One work viz *Cowles* (1) reports a bacteriophage which is active against *Cl tetani*, but this is said to have no influence on the toxigenicity Recently *Prescott & Altenbern* (16) attempted unsuccessfully to isolate bacteriophages from *Cl tetani* after induced lysis

It is thus still an open question whether a highly toxigenic gen residing in a temperate phage or some other transformation system are involved

The results reported in the present paper demonstrate that the highly toxigenic state of *Cl tetani* strain No 43415 is a labile phase to a certain degree and that maintenance of the high toxigenicity on subculture is dependent on the composition of the medium and the number of transfers

The stability of the toxigenicity is best in medium I in which medium the strain grows fast and copiously It is possible to make more than 50 transfers over a period of 3 to 5 months in that medium without any loss in toxigenicity whereas if subculture is carried out in medium II the toxigenicity of the strain will decrease to a very low level after about 30 transfers It has not been possible to regenerate the toxigenicity by the number of transfers in medium I carried out in this work

It should be pointed out that there must be an important difference in the metabolism during growth in these two media since the strain does not produce any or only very small amounts of toxin in medium I but an ample amount in medium II

Furthermore during consecutive transfers in medium I some metabolic change takes place in the cells since the toxigenicity decreases more rapidly by subsequent transfers in medium II About 30 transfers in medium II are needed to cause a gradual decrease to a very low toxigenicity whereas after a number of preceding transfers in medium I this decrease takes place after only two to three transfers

The experiments reported here also demonstrate that the selection of the low toxigenic mutant (or mutants) must be influenced by factors in the medium Selection may be caused either by a biochemical mutation such as a change in the enzymatic systems or by influencing the cell wall receptor mechanism

The improved anaerobic conditions caused by agar and thioglycollic acid did not seem to influence the decrease in toxigenicity

It is to be hoped that some of these problems together with other problems in this field may be elucidated in a better and less time consuming way by continued cultivation experiments Such experiments are in progress at present and the results will be published later

SUMMARY

1 The maintenance of the high toxigenicity of *Clostridium tetani* strain No 43415 in subcultures is dependent on the composition of the medium

2 The toxigenicity of the strain can be maintained for more than 50 transfers over a period of five months in a special peptone meat infusion medium with thioglycollic acid

3 Subculture of the strain in the *Mueller & Miller* medium used for routine production of tetanus toxin reduces its toxigenicity to a very low level after about 30 transfers

4 It was not possible by more than 200 consecutive subcultures in special peptone meat infusion medium with thioglycollic acid carried out over a period of two years to regenerate the toxigenicity of a culture which had lost about two thirds of its toxigenicity by subculture in *Mueller & Miller medium*

5 After consecutive transfers of the strain in special peptone meat infusion medium with thioglycollic acid the decrease in toxigenicity by subsequent transfer in *Mueller & Miller* medium occurred more rapidly

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19 *Soda S Yamato A Ito A & Murata R* Note on the maintenance of toxigenicity of seed cultures of *Clostridium perfringens* PB6K with special reference to alpha toxin Jap J Med Sci Biol *21* 91–94 1968

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BRIEF REPORTS

MILLIPORE® FILTER AS SUPPORT FOR FRESH FROZEN SECTIONS OF PERORAL BIOPSY SPECIMENS OF SMALL INTESTINE

By *Clas G Lindström*

If peroral biopsy specimens of small intestine are to be fixed (e g in Bouin s fluid) sectioned and stained the specimen may be placed directly on a firm support such as a fine mesh plastic net from which it can be removed before it is sectioned

But if frozen sections of unfixed biopsies are to be prepared for histochemical staining for enzymes for example it is not possible to use such a net or the like An ideal material should provide sufficient support for the specimen and it should be possible to cut it together with the specimen if the latter is to be sectioned parallel to the villi and perpendicular to the luminal surface So far however any *suitable material fulfilling these requirements is apparently not known*

Filter paper is not suitable because the cellulose fibres split when the paper is cut on the microtom with the result that the histological preparations will be uneven Agar and gelatine have also proved unsuitable because they are hard and difficult to cut when frozen besides which the sections are uneven and liable to split

It was therefore decided to try MF Millipore® filter with a pore size of 5 µ With this material as a support and the use of a suitable embedding medium for cryostat operation e g OCT® (Ames LAB TEK) during the actual freezing of the specimen

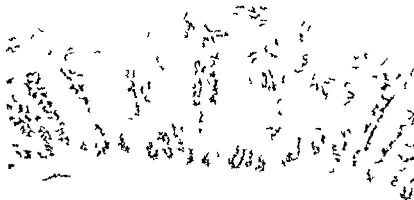


Fig 1

Biopsy from a 3 year old boy with coeliac disease treated with gluten free food Mounted on Millipore filter Fresh frozen section Succinic dehydrogenase × 7: Note the good orientation of the crypts of Lieberkühn

Received 6 x 69 from the University Department of Pathology University of Lund General Hospital 214 01 Malmö Sweden

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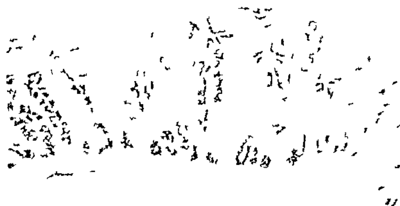


Fig 1

Biopsy from a 3 year old boy with coeliac disease trea[illegible] Mounted on Millipore filter Fresh frozen section Su[illegible] Note the good orientation of the cry[illegible]

Received 6 x 69 *from the University Department* [illegible] *General Hospital 214 01 Malmo Sweden*

Results and Discussion

Duodenum is rapidly labelled by leaking locally injected ^{3}H thymidine because of the rapid proliferation rate of the intestinal epithelial cells This labelling exceeds greatly the amounts of tritiated DNA brought there by labelled lymphoid cells During the 48 hour period from the labelling to the death of the animals extensive re utilization of tritiated DNA does not occur Therefore the ratio between spec act of the locally labelled organ and spec act of duodenum is an expression of the efficiency of the local labelling These ratios were compared with the corresponding ratios in the intravenously labelled animals by Student s t test (Table 1) Good local labelling was obtained both for thymus and bursa in all age groups of animals Autoradiography of the locally labelled organs showed high frequency of heavily labelled lymphoid cells mostly small lymphocytes Some heavily labelled large and medium sized lymphocytes could also be found In the thymus the heavily labelled cells were most frequent in the cortex and in the bursa the heavily labelled cells were rather uniformly distributed in the follicles

This method can be used for tracing of thymus and bursa derived cells in the other (lymphoid) organs of the normal chicken Results of such studies have been reported in part (*Linna et al* 1968 1969) and will be reported more extensively (*Hemmingsson & Linna* to be published) The method can also be used to study cell emigration from central lymphoid organs in chickens with stimulation or defects of the immune system

TABLE 1

Efficiency of Local Labelling of Bursa and Thymus with ^{3}H Thymidine Compared with Incorporation of Intravenously Administered Isotope into these Organs

Ratio	Way of labelling	Age of animals at labelling 24 hours	6 weeks	3 ½ months
spec act bursa / spec act duod	intrabursal	12 0 ± 2 0	16 0 ± 2 0	11 0 ± 2 7
	intravenous	0 35 ± 0 02	0 42 ± 0 03	0 50 ± 0 06
		p < 0 001	p < 0 001	p < 0 005
spec act thymus / spec act duod	intrathymic	4 0 ± 0 4	11 0 ± 2 0	10 0 ± 3 3
	intravenous	0 19 ± 0 02	0 14 ± 0 01	0 19 ± 0 01
		p < 0 001	p < 0 001	p < 0 005

Mean ± standard error of the mean

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ISOLATION OF CITRININ AND OXALIC ACID FROM *PENICILLIUM VIRIDICATUM* WESTLING AND THEIR NEPHROTOXICITY IN RATS AND PIGS

By *P Friis E Hasselager* and *P Krogh*

Nephrotoxic compounds were isolated from a strain of *Penicillium viridicatum* Westling This strain was isolated from a batch of barley which by feeding caused chronic kidney degeneration in pigs and rats (*Krogh & Hasselager* 1968) Liquid corn steep medium was inoculated and incubated as still culture for 30 days at 25 C followed by cold incubation at 5 C for 14 days After concentration of the liquid medium fractionation and isolation of nephrotoxic compounds was carried out using white Wistar rats as test animals Water suspensions of the fractions and compounds were administered perorally to rats over a period of 2–4 weeks

Two compounds were found nephrotoxic Citrinin and oxalic acid The citrinin induced kidney damage is characterized by enlarged kidneys histologically hydropic degeneration loss of brush border and pyknotic nuclei are observed in the proximal tubules These lesions are accompanied by thickening of the tubular basement membranes activation of interstitial cells and formation of collagen Some tubules are cystic dilated

This kidney degeneration is comparable to the kidney damage in rats and pigs caused by feeding barley inoculated with the strain of *P viridicatum* as well as by the batch of barley from which the fungus was isolated

In rats long term administration *per os* of oxalic acid and oxalates causes a chronic kidney damage characterized by dilated tubules formation of cysts and connective tissue and scattered crystals of calcium oxalate in the tubules No nuclear alterations were observed

The effect of the nephrotoxic compounds perorally administered to pigs was also studied

EXPERIMENT 1

Administration of Sodium Oxalate

Two pigs of 40 kg body weight received perorally administered sodium oxalate daily 200 and 1000 mg/kg body weight respectively for 42 days The oxalate was mixed with the feed which was readily eaten by the pigs No influence on the growth rate behaviour water consumption serum creatinine and blood urea nitrogen was observed By necropsy of the pigs and subsequent histological examination of all organs only a slight interstitial fibrosis was observed in the kidneys of the pig administered daily 1000 mg sodium oxalate/kg body weight Thus sodium oxalate seems to possess a very low toxicity to pigs

EXPERIMENT 2

Administration of Citrinin

Six pigs of 30 kg body weight received perorally administered citrinin which was mixed with the feed covered by an agar film The administration of citrinin was carried out daily according to the following schedule Two pigs received 20 mg/kg body weight during 34 and 70 days respectively two pigs received 40 mg/kg body weight during 8 and 42 days respectively and two pigs received 100 mg/kg body

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weight in 1 and 2 days respectively The last 2 pigs died in coma with renal insufficiency and 2500–2900 µg urea per ml plasma Accordingly the creatinine content was increased and a high glucoscuria was observed One pig which received 40 mg/kg for 8 days was killed because of feed refusal whereas decreased appetite was observed among the other pigs

At the level of 20 and 40 mg citrinin/kg body weight growth depression loss of weight and glucoscuria was observed whereas only a slight proteinuria was present Characteristically a strong increase of blood urea and blood creatinine was observed with a maximum after one week 3 times the normal value in pigs receiving 20 mg citrinin/kg and 5 times the normal value in pigs receiving 40 mg citrinin/kg In the urine sediment a big amount of large tubular epithelial cells with large pale nuclei was observed

At necropsy and subsequent histological examination the only alterations were found in the kidneys They were enlarged as regards the pig that received 40 mg citrinin/kg during 42 days 5 times the normal size The kidneys were grey yellow with a large number of small cysts under the surface Histologically the damage is characterized by a chronic degeneration of the proximal tubules especially in the last two thirds of the convoluted portion with development of atypical basophilic epithelium with enlarged polygonated nuclei with marked chromatin

The cysts are dilated tubular segments Thickening of the tubular basement membranes activation of the interstitial cells and formation of connective tissue was also observed

In Danish pigs the kidney daria is comparable to the chronic kidney degeneration caused by feeding mouldy grain as described by *Larsen* (1928)

Thus it is assumed that the kidney degeneration naturally occuring among Danish pigs is caused by citrinin although a synergistic effect with other compounds might exist.

A full report will be published later

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The Institute of Pathology II University of Umeå S 901 87 Umeå 6 Sweden the Division of Research Sinai Hospital of Detroit Detroit Mich 48235 USA and the Department of Anatomy University of Ibadan Ibadan Nigeria

SOME HISTOLOGICAL HISTOCHEMICAL AND ULTRA-STRUCTURAL STUDIES AND HORMONE ASSAYS IN A TRANSPLANTABLE ISLET CARCINOMA OF THE SYRIAN HAMSTER

By

STURE FALKMER LENNART BOQUIST PIERO P FOÀ T ADESANYA I GRILLO DOROTHEA L BAXTER GRILLO JEAN CLAUDE SODOYEZ FRANÇOISE SODOYEZ GOFFAUX and ALBERT J WHITTY

Received 16 v 69

The pancreatic islets contain four types of parenchymal cells three of them are granulated (α or D α or A and β cells) and one is agranular and probably identical to the γ cell of *Bowie* (1921) On the basis of histochemical and autoradiographic studies (*Fallmer et al* 1964) and of experimental and ultrastructural investigations with various kinds of regenerating islet tissue (*Boquist* 1968 a and b *Fallmer & Bergdahl* 1967) it has been suggested that the agranular cells are precursors of the granulated cells notably of the β cells (*Boquist & Falkmer* 1969b) Agranular cells are numerous in poorly differentiated human islet cell tumours (*Greider & Elliott* 1964 *Fallmer & Bergdahl* 1967 *Boquist & Fallmer* 1969a) and according to preliminary observations (*Boquist & Falkmer* 1969b) may be identical to the non granulated or sparsely granulated cells described by *Simar et al* (1968) in a transplantable islet cell carcinoma of the hamster We thought that a more detailed study of the morphological characteristics of this tumour and of the possibility that the tumour which has been shown to produce insulin (*Grillo et al* 1967 *Sodoyez et al* 1967) may produce other biologically active substances such as pro insulin gastrin secretin mono amines and mono amine oxidase could help elucidate the physiological role of the agranular cells

Request for reprints shall be sent to Professor Sture Falkmer Institute of Pathology II University of Umeå S 901 87 Umeå 6 Sweden

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Jean Claude Sodoyez is an Aspirant of the Fonds National de la Recherche Scientifique Belgium

MATERIALS AND METHODS

Tumour Transplants

Intramuscular and subcutaneous transplants of the tumour were prepared in adult Syrian hamsters (*Mesocricetus auratus*) as described previously (*Grillo et al* 1967) The samples of tumour tissue were excised after 3 to 9 weeks of growth from living anaesthetized animals and following the removal of grossly necrotic parts they were processed as described in each of the following sections

Light Microscopy

Specimens of tumour tissue fixed in Bouin s or Helly s fluids and embedded in paraffin were used for the common granule staining methods for islet cells and for silver impregnation by a new silver nitrate procedure (*Grimelius* 1968) and by a modification of Davenport s method (*cf Hellman & Hellerstrom* 1969) Heavy metals were detected using the sulphide silver procedure of Timm and Voigt (*Fallmer et al* 1964) in fresh frozen sections with H_2S and in paraffin embedded material fixed in H_2S saturated 70 per cent ethanol Biogenic amines were identified by fluorescence microscopy (*Falck et al* 1962 *Cegrell* 1969 *Cegrell et al* 1969) For this purpose pieces of tissue were lyophilized exposed to dry formaldehyde gas at 80 C for 3 hours embedded in paraffin cut into sections of 8 to 10 micra After removing the paraffin the sections were examined with a fluorescence microscope

Electron Microscopy

The tissue was prepared for electron microscopy according to the procedures described by *Boquist* (1967) and *Pihl* (1968) The former was used for conventional fine structure analysis the latter for the demonstration of heavy metals using a modification of the sulphide silver method

Atomic Absorption Spectrophotometry

The occurrence of heavy metals notably zinc and cobalt was checked by atomic absorption spectrophotometry (*cf Havu* 1969) Biopsy specimens from the tumour the thigh muscles of the opposite side the pancreas and the myocardium were taken from each of 5 to 8 hamsters the specimens were pooled and stored at 80 C

Determination of Pro insulin[1]

About 10 g of freshly excised tumour tissue were minced blotted on filter paper packed in dry ice and mailed to Chicago where 150 mg of tumour tissue were extracted with acid ethanol and fractionated on a column of Sephadex G 50 medium in 1 M acetic acid The fractions were collected in albumin coated tubes evaporated to dryness and dissolved in a buffer consisting of 0 1 M tris HCl pH 8 1 0 05 M sodium chloride and 0 25 per cent bovine serum albumin Immuno assay of the fractions was carried out using an antiserum to crude bovine pro insulin which reacts similarly with bovine pro insulin and insulin (*cf Steiner et al* 1969)

Assay for Gastrin

Gastrin was assayed in two laboratories using slightly different methods In one laboratory four batches of pooled tumour tissue weighing 16 6 to 35 8 g and one batch of fresh hog gastric mucosa weighing 1 4 kg were treated according to the method of *Gregory & Tracy* (1961) to make extracts equivalent to 0 7–15 g of tissue per ml All steps (except boiling) were carried out in the cold The gastric secretagogue activity of the extracts was tested in five rats using the technique described by *Lai* (1964) Each animal received injections of saline and of extracts of tumour and gastric mucosa in varying order In the other laboratory[2] about 5 g of pooled tumour tissue were collected as described above frozen at 40 C within ½–1 minute after excision packed in dry ice and mailed to Newcastle upon

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[1] kindly performed by Dr *Donald F Steiner* Department of Biochemistry University of Chicago

[2] This gastrin assay was kindly performed by Dr *Eric I Blair* Department of Physiology University of Newcastle upon Tyne

Tyn Here two samples weighing 1 3 and 1 1 g were broken up in a loosely fitting Potter Elvehjem homogenizer rotating at 660 r p m in approximately 4 volumes of water The homogenates were placed in a boiling water bath for 3 minutes and centrifuged Aliquots of supernatant fluid equivalent to 0 3 0 9 and 1 1 g of tumour tissue were assayed for gastrin activity by measuring their ability to stimulate acid secretion by the stomach of the anaesthetized cat (*cf Blair et al* 1969) The experimental procedure was controlled using 2 0 g of tumour tissue from a case of Zollinger Ellison syndrome (*Jalson et al* 1963) This tumour tissue had been stored at 18 C for 5 years

Assay for Secretin[3]

Two samples of tumour tissue weighing about 5 g each were used for secretin assay In addition the small intestine of 4 anaesthetized tumour bearing hamsters was excised cut up and freed from intestinal contents adjacent fat and blood The fresh material was minced immersed within 2 minutes into vigorously boiling de ionized water (about 3 g in 20 ml water) and boiled for 8–10 minutes (*Mutt* 1959) After cooling the tissue specimens were collected by filtering and resuspended in approximately 0 5 N acetic acid Glacial acetic acid was added to the filtrate in an amount sufficient to make a solution approximately 0 5 N Both fractions were saved and carried either in dry i e or at room temperature to Stockholm Here crude secretin was prepared within a week after collection of the material by adsorption on alginic acid elution with 0 2 N HCl and in some experiments precipitation with NaCl (*Mutt* 1959) The activity of the eluates was assessed in diluted aliquots (1 ml corresponding to about 0 5 or 1 0 g of wet tissue) by measuring the secretion of pancreatic juice in anaesthetized cats (*Mutt & Söderberg* 1959) A solution in physiological saline of pure porcine secretin containing 10 clinical units per ml was used as standard

Assay for Mono amine Oxidase

The presence of mono amine oxidase (MAO) was demonstrated histochemically by the reduction of tetrazolium salts (*Glenner et al* 1957) and chemically by the conversion of ^{14}C labelled 5 hydroxytryptamine (5 HT) to labelled 5 hydroxy indoleacetic acid (HIAA) according to the method of *Baker* (1966) For this purpose the tissue was homogenized (10 per cent w/v) in cold 0 1 M phosphate buffer pH 7 4 Aliquots of 0 2 ml were transferred to 5 ml centrifuge tubes kept in an ice bath 0 01 ml of labelled 5 HT creatine sulphate solution in distilled water (Radiochemical Centre Amersham specific activity 98 mc/mg 1 mg/ml) was added to each tube and the mixture was incubated at 37 C pH 7 4 for 20 minutes After incubation 0 15 ml of 1 N HCl and 3 ml of diethyl ether were added and the tubes were stoppered shaken and centrifuged at 2 00 r p m for 15 minutes at 0 C Two ml aliquots of the ether phase were pipetted into planchettes allowed to dry and their radioactivity was measured in a gas flow counter Control homogenates were heated to the boiling point cooled and incubated with the substrate

Assay for Proteolytic Enzymes

Four pieces of approximately 50 mg of tumour tissue were added to each of 20 Erlenmeyer flask containing 5 ml of Krebs Ringer bicarbonate buffer enriched with albumin (Fraction V Armour Pharmaceutical Co 0 5 per cent) and glucose (150 mg per cent) After incubation at 37 C in a Dubnoff metabolic shaker for 1 hour the pieces of tumour were removed by centrifugation the supernatant fractions were pooled and labelled insulin or labelled glucagon was added The mixture was then divided into 20 ml portions as follows a control portion containing an equal volume of a 0 5 per cent solution of albumin in phosphate buffer pH 7 4 and 5 experimental portions containing insulin (Iletin Lilly lot SG 1780-AMS) proteolytic enzyme inhibitor (Trasylol FBA Pharmaceuticals Inc) bovine albumin ACTH (Parke Davis and C # 35971) or glucagon (Lilly lot SF 8446-AMS) After standing at room temperature for various intervals of time 0 5 ml samples were removed from each flask mixed with 0 5 ml of a 10 per cent solution

[3] Kindly performed by Dr *Viktor Mutt* Gastrointestinal Hormone Research Chemistry Department Karolinska Institutet Stockholm

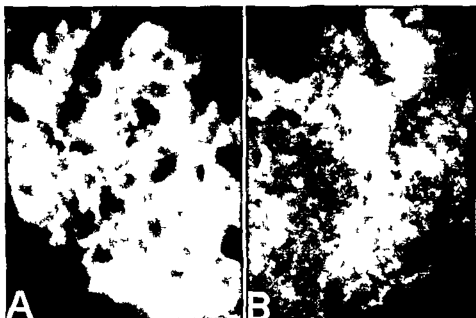


Fig 1

Green (A) and slightly yellowish (B) fluorescence of tumour tissue after exposure to formaldehyde gas indicating the presence of biogenic monoamines × 400

of trichloracetic acid (TCA) and centrifuged The precipitate was washed with an equal volume of 5 per cent TCA and its radioactivity was counted and expressed as per cent of the initial radioactivity

RESULTS

Light Microscopy

The most common tumour cells did not stain with aldehyde fuchsin showed no metachromasia with pseudo isocyanin and were devoid of secretory granules clearly identifiable by light microscopy None of the pseudo isocyanin negative cells were argyrophilic by either of the two silver impregnation methods or showed other tinctorial or histochemical characteristics of the α_1 or the α cells The second most common tumour cells were faintly aldehyde fuchsin positive contained sparse cytoplasmic granulation showing pseudo isocyanin metachromasia and for these reasons were classified as β cells No heavy metals could be detected with either variant of the sulphide silver method in the pseudo isocyanin negative cells or in the β cells

Catechol and Indole Derivatives and Mono amine Oxidase Activity

The production of a green sometimes slightly yellowish fluorescence following treatment with formaldehyde gas suggested that the tumour contained biogenic monoamines (Fig 1) From preceding chemical analyses (*Cegrell et al* 1969) it is known that this fluorescence es



Fig 2

Tetrazolium reduction suggesting mono amine oxidase activity A tumour tissue B pancreas of the lizard *Agama agama* islet of Langerhans surrounded by acinar tissue × 250

sentially is produced by a hitherto unidentified possibly monoamine like substance and also by dopa dopamine and 5 HT Fig 2 demonstrates the reduction of tetrazolium salts by tumour tissue and by a normal pancreatic islet and the relatively weak activity of the acinar tissue Table 1 shows that a marked MAO activity was found in tumour tissue and that this activity was almost three fold greater than that of normal hamster pancreas slightly higher than that of adult rat pancreas but lower than that found in the pancreas of rat and human foetus and of chick embryos

TABLE 1

Assay for the Activity of Mono amine Oxidase in Homogenates of Pancreas and in Islet Cell Tumour Radioactivity of 5 hydroxyindoleacetic Acid Obtained Following Incubation with ^{14}C Labelled Serotonin (Counts/min/mg of Dry Weight)

Tissues	Untreated Homogenate	Heated Homogenate
Hamster Tumour	14 956 ± 2529	1 634 ± 467 P < 0 01
Hamster Pancreas	5 034 ± 4 1	1 058 ± 287 P < 0 01
Rat Pancreas	10 562 ± 168	2 153 ± 931 P < 0 01
Pancreas of a 17 Day Chick Embryo	2 964 ± 1162	2 546 ± 461 P < 0 001
Pancreas of a 133 mm Human Foetus	20 04 ± 3698	1 769 ± 249 P < 0 01
Pancreas of a 22 Day Rat Embryo	18 956 ± 845	761 ± 273 P < 0 001



Fig 3

Low power electron micrograph of the cells of the transplantable Syrian hamster islet carcinoma, showing a predominance of agranular or only sparsely granulated tumour cells The nuclei are of varying shape sometimes with identations The cytoplasm is electron translucent Several large mitochondria and lysosomal dense bodies are seen × 3 000

Electron Microscopy

The most abundant cells were polygonal with a rather electron transparent cytoplasm and a large nucleus often showing indentations

(Figs 3 and 4) Mitochondria varied in number and were frequently abundant the Golgi apparatus was usually prominent Most of these cells seemed to lack characteristic secretory granules although several of them with otherwise identical ultrastructural features contained occasional round granules of the β type (Figs 4 and 5) Several kinds of cytoplasmic bodies were observed some of them were peculiarly shaped and apparently related to mitochondria and lysosomal dense bodies The endoplasmic reticulum was both smooth and rough and moderately prominent often forming clear round vacuoles Parasomes were rare free ribosomes moderately frequent Cells with occasional β like secretory granules sometimes contained basal bodies of cilia as well as one or more bundles of tonofilaments Except for the few β granules the ultrastructural characteristics of the cells identified as β cells by light microscopy were essentially those described by *Simar et al* (1968) No α_1 or α cells were found The modified sulphide silver procedure for detecting heavy metals gave practically negative results both in the agranular or sparsely granulated tumour cells and in the tumour cells with β granules

Atomic Absorption Spectrophotometry

The average zinc content of tumour tissue was 2 1 mg/100 g wet weight that of the 3 other hamster tissues assayed in parallel was of the same order of magnitude Likewise no cobalt content significantly higher than in the 3 other hamster tissues was observed in the tumour

Proinsulin

Two peaks of immunoreactive material with the characteristic positions of pro insulin and insulin were detected in the fractions obtained from the Sephadex column the early pro insulin peak represented 20 per cent of the total immunological activity Adsorption of the antiserum with purified bovine insulin significantly enhanced its ability to react with early peak material suggesting that this material was pro insulin or a related substance

Assay for Gastrin

Fig 6 shows that no evidence for the presence of gastrin in tumour tissue could be obtained using the first extraction procedure and the rat assay method although hog gastrin produced the expected stimulation of HCl secretion Tumour extracts prepared and assayed according to the second procedure and equivalent to 0 2 and 0 9 g of tissue showed no detectable gastrin activity The extract of 1 1 g of tissue contained the smallest activity detectable by the assay which is sensitive to 25 micromoles (or 50 nanograms) of synthetic human gastrin I (*cf Blair et al* 1969) On the other hand the control injection

TABLE 2

Assay for the Presence of Secretin in HCl Eluates from Boiled Hamster Islet Carcinoma and Intestine Extracted with 0.5 N Acetic and Adsorbed on Alginic Acid

Order of injection	Preparation	Dose	Pancreatic response (μ eq alkali)
1	Purified porcine secretin	1 clin unit	280
2	Extract of hamster gut	1 0 ml	190
3	Physiological saline	1 0 ml	0
4	Extract of hamster gut	1 5 ml	160
5	Extract of hamster tumour	1 0 ml	40
6	Extract of hamster tumour	1 5 ml	30
7	Purified porcine secretin	1 clin unit	340

The amounts of pancreatic juice secreted by an anaesthetized cat after intra venous injection of the diluted eluates were expressed as micro equivalents of alkali

The effects of the eluates were compared with those of purified porcine secretin and the injections were made in the order indicated in the Table

The dilution was adjusted so that 1 ml corresponded to 1 g wet weight of boiled tissue

Proteolytic Enzymes Assay

Figs 7 and 8 show that labelled insulin added to the medium which had been used for the incubation of tumour tissue was degraded at a rapid rate and could no longer be precipitated by TCA Trasylol and ACTH provided relatively little protection against insulin degradation whereas bovine albumin insulin and glucagon effectively protected the labelled hormone Fig 9 shows that glucagon was also very sensitive to the proteolytic enzymes released by the tumour and was partially protected by insulin glucagon and ACTH

TABLE 3

Assay for the Presence of Secretin in the Sodium Chloride Precipitate of the HCl Eluates

Order of injection	Preparation	Dose	Pancreatic response (μ eq alkali)
1	Purified porcine secretion	1 clin unit	340
2	NaCl precipitate of hamster gut eluate	5 g	100
3	Same as No 2	10 g	130
4	NaCl precipitate of hamster tumour eluate	5 g	10
5	Same as No 4	10 g	0
6	Same as No 2	10 g	125
7	Same as No 1	1 clin unit	300

Estimated amount of tissue represented by the volume injected
Preparation of the eluates and other experimental conditions as in Table 1

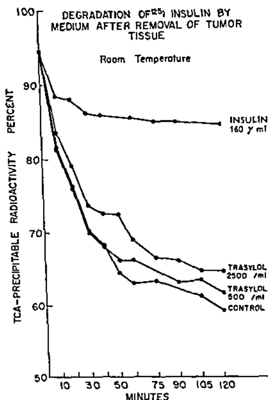


Fig 7

Degradation of 12 I labelled insulin by buffer in which tumour tissue had been incubated for 1 hour Effect of Trasylol and of non labelled insulin

DISCUSSION

The results of the light microscopic histochemical and ultrastructural investigations described in this paper confirm and extend those reported previously (*Grillo et al* 1967 *Sodoyez et al* 1967 *Simar et al* 1968) The rare occurrence of β granules in the tumour cells the negative reaction obtained with all variants of the sulphide silver procedure for the histochemical demonstration of heavy metals and the failure to find zinc or cobalt in amounts larger than those found in other tissues provide additional evidence of the low insulin content of the tumour It should be pointed out that heavy metals notably zinc and possibly also cobalt (*Havu* 1969) occur in the secretory granules of the β cells of most species (*Pihl & Falkmer* 1967) and that they probably play a role in the storage of insulin (*Falkmer & Pihl* 1968) The criteria used for the designation agranular islet cell are (*Falkmer et al* 1964 *Boquist* 1969) that the differential staining procedures for islet tissue shall give only a non characteristic background colour that the sulphide silver proced

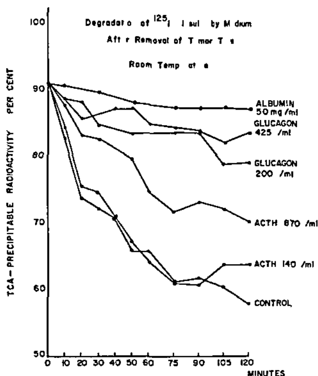


Fig 8

Degradation of 1 51 lab lled insulin by buffer in which tumour tissue had been incubated for 1 hour Effect of ACTH glucagon and albumin

be polygonal and have a rather prominent nucleus of varying shape sometimes with indentations and that the cells shall have an electron translucent cytoplasm and a variety of cytoplasmic organelles Usually these organelles are only moderately conspicuous but in some cases lil e in the cells of the hamster tumour mitochondria and Golgi ap paratus are prominent the latter sometimes showing small primitive secretory granules apparently of the β type Thus the tumour appears to be constituted primarily of agranular or sparsely granulated cells and should be a good object for investigating their biological proper ties The presence of completely agranular cells (Fig 3) of granulated cells of the β type (Fig 5) and of an intermediate type of sparsely granulated cells with ultrastructural characteristics of both (Fig 4) supports the hypothesis that the agranular cells represent immature β cells or β cell precursors (*Boquist & Fallmer* 1969a and b *Boquist* 1969) The fact that the tumour contains less totaly extractable im munoreactive insulin (*Sodoyez et al* 1967) and relatively more pro insulin than normal pancreas just as human islet cell tumours do (*Steiner et al* 1969) suggests the intriguing possibility that the pro β cells may be involved in the synthesis of pro insulin Indeed the transformation of agranular into granulated cells is very similar to

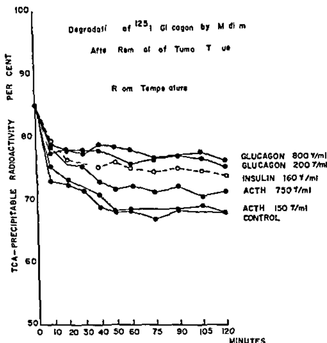


Fig 9

Degradation of 125I labelled glucagon by buffer in which tumour tissue had been incubated for 1 hour Effect of ACTH insulin and non labelled glucagon

the transformation occurring in the normal islets during embryologic development (*Grillo* 1964 *Wells et al* 1968) at the time when measurable amounts of insulin begin to be synthesized (*Wessells & Rutter* 1969)

Our failure to demonstrate secretin or gastrin in tumour extracts as well as the failure to find evidence of gastrin production by the agranular cells of the islet parenchyma of lower vertebrates (*Blair et al* 1969) leaves the cellular origin of these two hormones undetermined There are however some recent reports that they may originate from granulated cells (*cf Demling & Ottenjann* 1969 *McGuigan* 1969)

The experiments described in this paper confirm the fact that the normal islets and the islet cell tumour contain biogenic amines (*Cegrell* 1969 *Cegrell et al* 1969 *Jaim Etcheberry & Ziehr* 1968 *Cession Fossion & Lefebvre* 1969) and MAO activity (*West* 1958) The role played by these substances in the pancreatic islets is unknown although they may mediate the glycogenolysis induced by anoxia (*Hellman & Idahl* 1969) the hyperglycaemic action of growth hormone and deserpidine (*Colombo et al* 1960 *Foa et al* 1953 *Galansino et al* 1960 *Sirek & Sirek* 1966) or the action of the autonomic nervous system on the pancreas The latter has been postulated on the basis of physiological (*Zunz & La Barre* 1927 *Daniel & Henderson* 1967) pharma

cological (*Kaneto et al* 1968 *Telib et al* 1968 *Cegrell* 1969 *Cegrell et al* 1969 *Nelson et al* 1967, *Kansal & Buse* 1967 *Porte* 1967) and histological (*Patent & Alfert* 1967 *Esterhuizen et al* 1968 *Morgan & Lobl* 1968) evidence The presence of biogenic amines in the hamster tumour and of MAO both in islets and in tumour tissue provides additional evidence of the similarity between this insulin producing tumour and the pancreatic islets from which it was derived The role of MAO in the biology of the tumour is a matter of speculation it is possible that the carbonyl derivative of serotonin produced by this enzymatic reaction may reduce oxygen uptake and P/O ratio (*Mahler & Humoller* 1968) thus decreasing the ratio of ATP to ADP and explaining at least in part the high rate of glycolysis characteristic of the tumour (*Sodoyez et al* 1969b)

The nature of the proteolytic enzymes released into the incubation medium by tumour tissue remains uncertain for they were not characterized biochemically Nevertheless the ineffectiveness of Trasylol a powerful kallicrein trypsin chymotrypsin inactivator (*Trautschold et al 1967*) suggests that the tumour enzymes are not related to those of the exocrine pancreas This hypothesis is supported by the fact that tumour cells do not contain zymogen granules (*Grillo et al* 1967 *Sumar et al* 1968) It should be pointed out that while normal islets do not release proteolytic enzymes into the incubation medium (*Malaisse et al* 1967 *Sodoyez et al* 1969a) malignant tumours do (*Sylven & Bois Svensson* 1965) The rapid proteolytic destruction of insulin and glucagon by tumour tissue *in vitro* may explain some of the difficulties encountered in the immunoassay of these hormones (*Sodoyez et al* 1969b *Nonaka et al* 1969)

SUMMARY

A transplantable insulin producing hamster islet carcinoma appeared to be constituted primarily of agranular or sparsely granulated cells and was consequently considered to be a good object for further investigations of the biological properties of these islet parenchymal cells Apart from agranular cells and β cells no other islet cells occurred in the tumour

The presence of sparsely granulated tumour cells with ultrastructural characteristics both of completely agranular cells and of granulated β cells supported the hypothesis that the agranular cells represent immature β cells or β cell precursors Further support for this hypothesis was obtained by the findings of less totaly extractable immunoreactive insulin and relatively more pro insulin in the tumour than in normal pancreas The zinc and cobalt contents were both insignificant

In addition to insulin and pro insulin the tumour cells were found to contain biogenic amines (catecholamines and 5 hydroxytryptamine)

and mono amine oxidase Moreover incubated tumour cells could release significant amounts of proteolytic enzymes
The islet carcinoma did not contain gastrin or secretin

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MODIFIED TECNIQUE OF MUSCLE BIOPSY

By

EDITH RESKE NIELSEN
AAGE HARMSEN and JACOB HØJGAARD

Received 21 v 69

The diagnostic significance of the muscle biopsy is constantly increasing not only in neuromuscular diseases but also within the neurosurgical field and in medical diseases e g diabetes mellitus and the collagen diseases

Muscle tissue is very sensitive to any stress Therefore the technical treatment of the biopsy is of the greatest importance to the later evaluation

The object of this work is to give a collected account of all the details concerning the technical procedures obtained on the basis of 320 muscle biopsies

Detailed descriptions will be given of the taking of biopsies the preparation of the material for light and electron microscopy and of a modification of *Koelle*'s histochemical method for demonstration of the subneural apparatus and of *Coers* method for demonstration of the intramuscular nervous system

The preparation of a muscle biopsy from the taking till the finished result is of great importance Therefore all procedures were performed by trained and well qualified people

TAKING OF SPECIMENS

The muscle biopsies are taken under the usual sterile precautions The operation is generally made in local anaesthesia (10–15 ml inj lidocain noradrenalin 0 5 per cent)

To prevent artifacts in the biopsy only skin and subcutis are infiltrated with the anaesthetic not the muscles

Only on rare occasions full anaesthesia is used e g in children or in restless nervous adults

A skin incision– 6 to 8 cm long— is placed longitudinally in the extremity and the muscle fascia is split in the fibre direction for about 6 cm Thus the biopsy may be taken in the whole length of the muscle fibres and it is not necessary to ensure the localization of the end plates by electro-myography on the muscle exposed

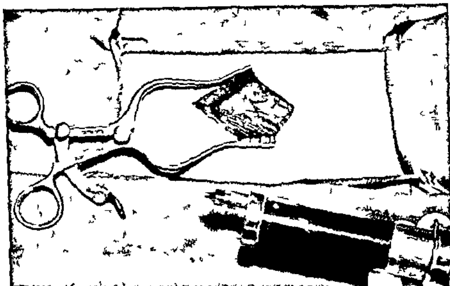


Fig 1

Biopsy of the anterior tibial muscle The fascial edges are kept apart by a self retaining retractor The exposed muscle is ready for injection of methylthionine *Note the bent needle*

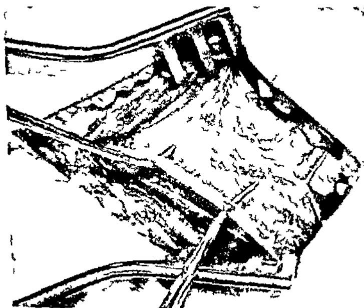


Fig 2

A muscle strip is taken for electron micr scopy by means of two single hooks

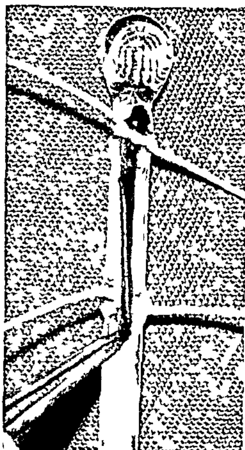


Fig 3
The muscle strip for electron microscopy is stretched

To obtain a good result of the examination it is very important that the muscle is manipulated as little as possible

With a self retaining retractor the fascial edges are kept apart (Fig 1) and the perimysium is dissected bluntly or incised

By means of two single hooks a muscle strip (1–2 mm thick) is first taken in the entire length of the fibre for electron microscopy (Fig 2) This strip is immediately stretched on a glass rod (Fig 3) by tying a ligature in each end These ligatures are fixed on the glass rod with dental wax so that the muscle fibre may be stretched 10–15 per cent By so doing all bands in the muscle fibrils stand out very clearly in the electron microscope The glass rod with the biopsy is placed in the fixative After the taking of the muscle for electron microscopy the exposed part of the selected muscle is infiltrated for at width and depth of 1 cm by 10 20 ml inj methylthionini chloridi 0 15 per mille

The dye is injected by a thin (gauge 11) bent needle inserted in the fibre direction at various depths The injection is made slowly and carefully both during the insertion and withdrawal of the needle until the muscle is maximumly blue (Fig 1)

5 minutes after the beginning of the injection the muscle piece in question is cut distally (¾–1 cm in the width and in the depth), carefully gripping the distal end of the muscle piece with a pair of tweezers and isolating it to the proximal end. Only the ends of the muscle piece must be gripped. With a pair of scissors 4–5 strips are cut from the whole length of the muscle piece and placed on gauze moistened with saline for demonstration of the intramuscular nervous system. One strip is placed in saline 0.9 per cent for demonstration of the subneural apparatus and one strip also placed in saline for light microscopy.

TREATMENT OF MUSCLE BIOPSY

With minimal modifications earlier described methods have been used. Careful treatment of the biopsy for paraffin wax sectioning is very important to avoid shrinking and artifacts. The following procedure is employed:

1 day: the muscle biopsy received in saline 0.9 per cent is placed on a cork with porcupine bristles (not metal needles) and stretched to its original length. It remains in saline for about 2 hours, then in formalin solution 4 per cent over night.

2 day: the biopsy is removed from the cork, washed in running water for about 8 hours and then placed in aethanol 70 per cent over night.

3 day: the biopsy is placed in aethanol 96 per cent which is changed once during the day.

4 day: the biopsy is placed in aethanol 99 per cent in a thermostat 37° C for about 8 hours and then in ligroine over night.

5 day: the specimen is cut transversely of fibres. The two strips are placed in pure ligroine where they remain for about 4 hours, then in paraffin wax in a 58° C hot oven.

6 day: embedded so that one piece is cut transversely, the other longitudinally.

7 day: the specimen is cut at 7 μ and stained.

The following general stainings are made on the paraffin wax section: haematoxylin eosin method, Van Gieson Hansen and toluidine blue. The following special stainings are made: PAS, Weil and Davenport (*Romeis* 1948).

According to the experience of the department the slow dehydration of the specimens and the clearing in ligroine are very important for obtaining good specimens.

DEMONSTRATION OF THE SUBNEURAL APPARATUS

The principle of the histochemical method is the exploitation of the content of acetylcholine esterase in the subneural apparatus (*Barka & Anderson* 1963).

The histochemical staining of the subneural apparatus is performed by a simplified and modified technique described by *Koelle & Friedenwald* (1949) modified by *Couteaux* (1951).

On the basis of *Koelle's* method two stable solutions A and B have been prepared and these two form the Incubating Medium (*Coërs & Woolf* 1959).

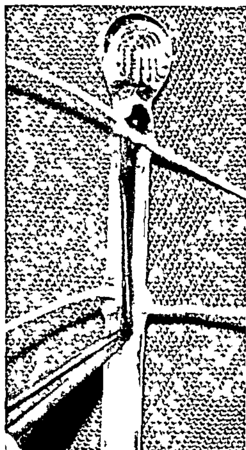


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The dye is injected by a thin (gauge 14) bent needle inserted in the fibre direction at various depths The injection is made slowly and carefully both during the insertion and withdrawal of the needle until the muscle is maximumly blue (Fig 1)

5 minutes after the beginning of the injection the muscle piece in question is cut distally (¾–1 cm in the width and in the depth) carefully gripping the distal end of the muscle piece with a pair of tweezers and isolating it to the proximal end Only the ends of the muscle piece must be gripped With a pair of scissors 4–5 strips are cut from the whole length of the muscle piece and placed on gauze moistened with saline for demonstration of the intramuscular nervous system One strip is placed in saline 0 9 per cent for demonstration of the subneural apparatus and one strip also placed in saline for light microscopy

TREATMENT OF MUSCLE BIOPSY

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Careful treatment of the biopsy for paraffin wax sectioning is very important to avoid shrinking and artifacts The following procedure is employed

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5 day the specimen is cut transversely of fibres The two strips are placed in pure ligroine where they remain for about 4 hours then in paraffin wax in a 58 C hot oven

6 day embedded so that one piece is cut transversely the other longitudinally

7 day the specimen is cut at 7 μ and stained

The following general stainings are made on the paraffin wax section haematoxylin eosin method Van Gieson Hansen and toluidine blue The following special stainings are made PAS Weil and Davenport (*Romeis* 1948)

According to the experience of the department the slow dehydration of the specimens and the clearing in ligroine are very important for obtaining good specimens

DEMONSTRATION OF THE SUBNEURAL APPARATUS

The principle of the histochemical method is the exploitation of the content of acetylcholine esterase in the subneural apparatus (*Barka & Anderson* 1963)

The histochemical staining of the subneural apparatus is performed by a simplified and modified technique described by *Koelle & Friedenwald* (1949) modified by *Couteaux* (1951)

On the basis of *Koelle*s method two stable solutions A and B have been prepared, and these two form the Incubating Medium (*Coers & Woolf* 1959).

Solution A is composed in the following way

Sodium acetate ($CH_3COONa\ 3H_2O$)	3 320 g
1 N acetic acid	0 f25 ml
Demineralized water to make	250 ml

pH is tested to 6 20 after which the solution is mixed with a solution consisting of

Cupri sulphate ($CuSO_4\ 5H_2O$)	250 mg
Aminoacetic acid	375 mg
Demineralized water to	210 ml

Solution A consists of *Koelle*s solution 1 + 2 + 3

Solution B also named solution medium consists of

Cupri sulphate ($CuSO_4\ 5H_2O$)	1 248 g
Demineralized water to make	200 ml

In a refrigerator (4 C) the solutions A and B will keep for 3 months

Before the incubating solution shall be prepared a sufficient amount of the solutions A and B is heated to 90 C–92 C

Incubating Solution

145 mg of ATHCH (acetylthiocholiniodide) is dissolved in 10 ml of solution B *and centrifuged for about 20 minutes The supernatant is filtrated and* 2 4 *ml of* the clear filtrate is mixed with 27 6 ml of solution A (must be prepared fresh day by day)

By this modification two stable liquids are obtained (solution A and solution B) which may be prepared at the local pharmacy as well as a stable substance ATHCH which will remain stable provided they are kept under proper conditions Thus the technical staff has only two solutions and one dry solid to work with which is a great relief

Subsequently the procedure is a follows

1 The muscle biopsy is kept in saline 0 9 per cent for one hour
2 Then in neutral 10 per cent formaldehyde solution for 1–2 hours
3 Longitudinal sectioning on freezing microtome 60–70 μ thick
4 Rinsed twice in distilled water
5 The sections are incubated at 37 C in the incubating solution At this enzymic reaction a copper thiocholine precipitate is formed and at every 15 minutes sections are taken out These go through the following procedure rapidly
6 Rinsed twice in distilled water
7 Placed in a 5 per cent ammonium sulphide solution for 1–1½ minute

By so doing the copper thiocholine precipitate is transformed into an amorphous copper sulphide by which the end plates become visible (The above procedure is continued until a suitable colour intensity is obtained)

8 The *specimens are rinsed* twice in distilled water
9 Dipped briefly into 3 per thousand toluidine blue (*F Christensen* 1959)
10 The sections are now mounted dehydrated and embedded

The sections are during the whole procedure manipulated with a glass hook

This modification of *Koelle s* staining is easily performed and renders excellent results (Fig 4)

Vital Staining of the Intramuscular Nervous System with Methylene Blue

The demonstration of the intramuscular nervous system is initiated with the intravital staining with methylthionine chloride The dye is

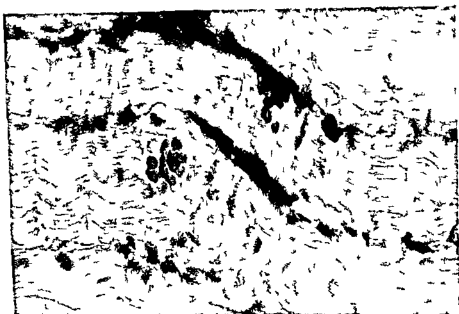


Fig 4

Normal subneural apparatuses in a anterior tibial muscle

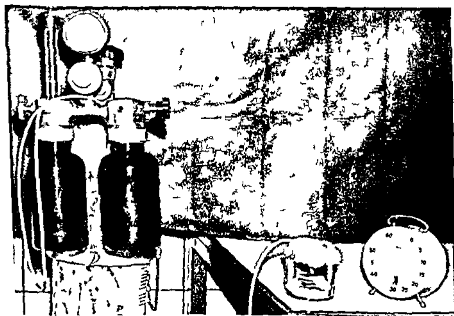
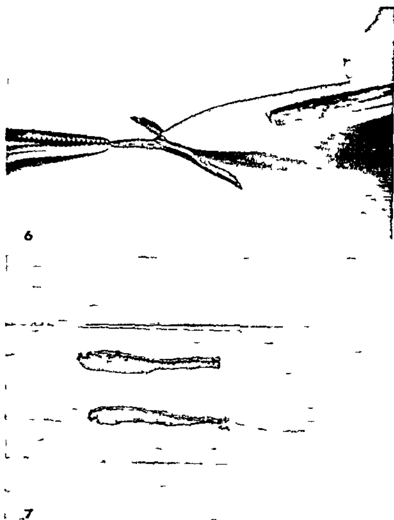


Fig 5

The muscle strips are placed under cover and oxygenated



Figs 6–7

Fig 6 A muscle strip is split longitudinally by a loose leaf scalpel
Fig 7 Muscle strips are squeezed between two slides

bound in the intramuscular nervous system and colours this At after treatment of the biopsy with oxygen the existing leucomethylthionine derivatives will be transformed into methylthionine derivatives

The preparation of the biopsy after the taking is a modification of *Coërs* method (*Coërs and Woolf* 1959 *Coërs* 1952a)

The procedure is performed in the following way

1 The muscle strips are placed on moistened gauze under cover and frequently sprinkled with saline 0 9 per cent The specimens are oxygenated for one hour in pure oxygen with a suitable flow (after 30 minutes the specimens are turned) (Fig 5)

2 The strips are now stretched to the original length on a cork with porcupine

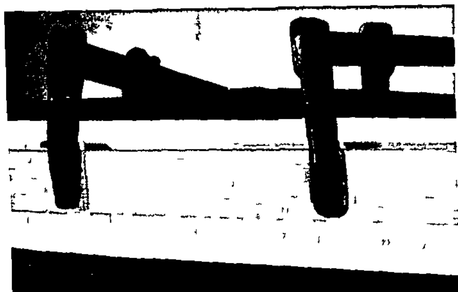


Fig 8

The slides are pressed between two even wooden pieces

bristles and fixed in a filtered cold aqueous saturated solution of ammonium molybdate at 4 C for twenty four hours

3 Then washed in three changes of distilled water 4 C

4 The strips are then split longitudinally by hand by a loose leaf scalpel at 80 μ placed between two slides and squeezed carefully (Fig 6 and 7)

5 The slides are kept in press between two even wooden pieces (Fig 8) by means of two screw clamps during about one hour

6 The slides are carefully separated

7 Manual dehydration of the specimens on the horizontally placed slides by means of a pipette

Twice for about 2 minutes in aethanol 70 per cent
Twice for about 2 minutes in aethanol 96 per cent
Twice for about 2 minutes in aethanol 99 per cent

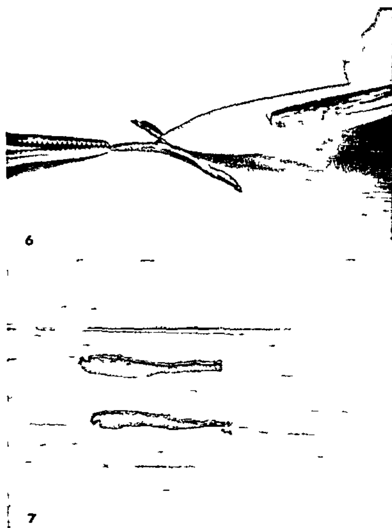
8 Clarification in toluene twice Then embedding in resin The hand made specimens render an extremely beautiful result with possibility for study as well as photography of the single end plates and of fibres and bundles

Contrary to *Coers* method hand cut preparations are used which give a good survey of bundles as well as of terminal axons In the last five cases the muscle strip is stretched after fixation with good result (Fig 9 A + B) According to our opinion this is more careful with the tissues than freeze cutting The simultaneous pressing of all preparations is a facilitation to the technical staff

The Preparation of Muscle Specimens for Electron Microscopy

The following solutions are needed

1 Fixative (*Pease* 1964)
4 ml of glutaric acid 25 per cent



Figs 6–7

Fig 6 A muscle strip is split longitudinally by a loose leaf scalpel
Fig 7 Muscle strips are squeezed between two slides

bound in the intramuscular nervous system and colours this At after treatment of the biopsy with oxygen the existing leucomethylthionine derivatives will be transformed into methylthionine derivatives

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The procedure is performed in the following way

1 The muscle strips are placed on moistened gauze under cover and frequently sprinkled with saline 0 9 per cent The specimens are oxygenated for one hour in pure oxygen with a suitable flow (after 30 minutes the specimens are turned) (Fig 5)

2 The strips are now stretched to the original length on a cork with porcupine



Fig 10

Capsules with imbedded specimens are placed in a plastic rack

4 Vestopal W
100 ml of Vestopal
1 ml of initiator
1 ml of activator

The Vestopal is stirred well with 1 ml of initiator for 5 minutes then 1 ml activator is added and the mixture is stirred well for 5 minutes

The preparation of the specimens for electron microscopy is then as follows

1 Fixation in glutaric aldehyde for 24 hours

2 Rinsing fluid for 24 hours

3 The muscle biopsy is removed from the glass rod the middle piece with the assumed end plates is divided into thin layers and further fixed in osmium tetroxide for one hour

4 Rinsing fluid for 24 hours

5 Dehydration

20 minutes in aethanol 24 per cent
20 minutes in aethanol 60 per cent
20 minutes in aethanol 62 per cent
20 minutes in aethanol 93 per cent
2 × 15 minutes in aethanol 99 per cent
2 × 45 minutes in 100 per cent acetone p a with dried copper sulphate (filtered before use)

6 The strips are placed in a beaker with a mixture of Vestopal 25 per cent and pure anhydrous acetone 75 per cent p a They remain uncovered in this solution for 24 hours in a thermostat at 30 C

7 As much as possible of the Vestopal is poured off and the beaker is filled with an identical amount of 100 per cent Vestopal This is left uncovered at 30 C for 24 hours

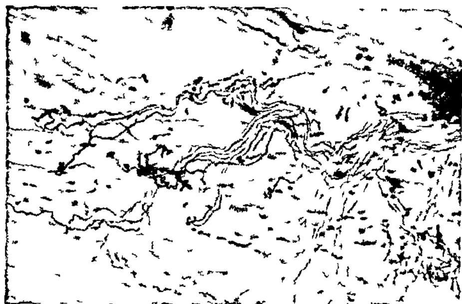
8 Embedding The specimens are placed at the bottom of small gelatine capsules by means of a thinly drawn out glass rod a small label with number and consecutive letters are placed inside at the top of the capsule with a pair of fine tweezers Then the capsules are filled with Vestopal by a normal eye pipette with cut off point

The capsules are placed in a plastic rack which may hold 20 capsules in all (Fig 10)

9 The specimens are placed in the oven for 24 hours at 30 C
24 hours at 40 C
48 hours at 60 C

10 Filing

The specimens may be cut 24 hours after the final preparation



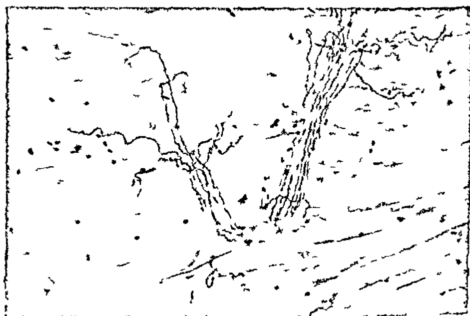


Fig 9 A and B

Nervebundles subterminal fibres and end plates in a stretched normal long palmar muscle A Low magnification B High magnification

20 ml of phosphate buffer pH 7.3
26 ml of distilled water
The ingredients are mixed well immediately before use
2 1 per cent isotonic osmium tetroxide solution (0 9 per cent saline)
3 *Rinsing fluid* 0 15 M *sucrose in* 0 075 M *phosphate buffer pH* 7 3

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CLEAR-CELL SARCOMA OF TENDONS

A Study of 4 Cases

By

LENNART ANGERVALL and BERTIL STENER

Received 23 vi 69

Malignant tumours arising from tendons are rare Among those reported most have been interpreted as synovial sarcoma or fibrosarcoma until *Enzinger* in 1965 described a new type which he called clear cell sarcoma of tendons and aponeuroses This tumour which is roughly spherical firm and usually well defined consists of compact nests and fascicles of pale staining round or fusiform cells of epithelioid appearance The cellular aggregates are enclosed by delicate fibrous septa merging with tendinous or aponeurotic tissue Most of the 21 tumours studied by *Enzinger* were located in the lower extremity the foot being the most common site The tumours had been collected in the files of the Armed Forces Institute of Pathology in Washington D C USA during 25 years Considering the large material of this institute these figures reflect the rarity of the tumour Additional information on clear cell sarcoma is given in the present paper The 4 tumours studied by us had been collected in the Departments of Pathology Sahlgren Hospital Goteborg during the last 20 years again an indication of the rarity of the tumour as these departments collect material from a part of Sweden inhabited by more than 1 million people

CASE REPORTS

Case 1 A man 39 had received a hard blow to the inner aspect of the left knee 2 years before he presented with a tumour in the same region During the last 3 months the tumour had increased rapidly in size and had become painful.

On examination a firm non fluctuant rounded tumour was felt posteromedial to the knee joint The circumference over the most prominent part of the tumour was 46 cm as compared with 34 cm on the other leg The mobility of the joint was greatly restricted The skin over the tumour felt warm Several enlarged lymph nodes (2–3 cm) were palpated in the left groin

An incisional biopsy (December 1950) was done and the pathologist s report was a synovial sarcoma Radiotherapy was given to the primary tumour (tumour dose 4000 R) without any decrease of its size and to the regional lymph nodes (skin dose 3300 R) with some regression as a result The patient died after 6

We are indebted to Professor *Ingmar Wickbom* for interpretation of the radiographic findings in Cases 3 and 4 and for allowing us to publish Fig 1

months with radiographic evidence of metastatic lesions in the lungs the lumbar spine and the pelvis

Case 2 A man 27 had noticed a slow growing painless mass in the right lower leg for 3 years

Examination revealed a tumour 7 × 5 cm b hind the tibial malleolus The tumour moved with the Achilles tendon during flexion and extension of the ankle joint

At operation (November 1960) the tumour was found to lie close to the Achilles tendon It was excised together with the overlying skin and the adjacent part of the tendon

Two years and 3 months after the operati n a lymph node enlargement 3 × 5 cm suspected to be a metastasis was palpated in the right groin A slight re gression was noticed following radiotherapy (skin dose 1500 R) An ilio inguinal lymph node dissection was then carried out Histological examination confirmed metastatic lesion in the palpated enlarged node

Three years after the excision of the primary tumour the patient died with metastatic lesions in retroperitoneal and mediastinal lymph nodes the skin the lungs the pleura the myocardium the pericardium the liver the peritoneum the omentum and the skeleton

Case 3 A man 28 had noticed a growing lump near the upper inner margin of the right patella for 1 year He had experienced pain in the lump while working with the knee bent

Examination showed a firm ovoid tumour 7 × 5 × 5 cm locatel at the mu sculotendinous junction of the vastus medialis The tumour moved with the patella on flexion and extension of the knee joint

Femoral angiography (Fig 1) showed that the tumour was highly vascular Many irregular tortuous vessels filled within the tumour some of them varying markedly in width moreover a diffuse opacification by contrast medium oc curred Veins draining the tumour filled while contrast medium still remained in the femoral artery indicating arterio venous shunting Angiographically the tumour appeared well defined

At operation (January 1961) the tumour was removed by a wide local excision including the whole of the vastus medialis part of the quadriceps tendon the upper medial third of the patella the fibrous capsule of the knee joint on the medial side with underlying synovial tissue and the long tendon of the adduct r magnus The tibial end of the sartorius was attached to the patella in order to prevent lateral dislocation Through this procedure the distal thirl of the sarto rius was given a direction similar t that of the removed vastus medialis

After the operation the patient returned to work as a plater At follow up 1 year later he could extend the knee joint fully and flex it 70 The transferrel sartorius muscle became activated during voluntary flexion of the knee joint but remained relaxed during extension The patient had not noticed any tendency of lateral dislocation of the patella nor could such be demonstrated at clinical examination

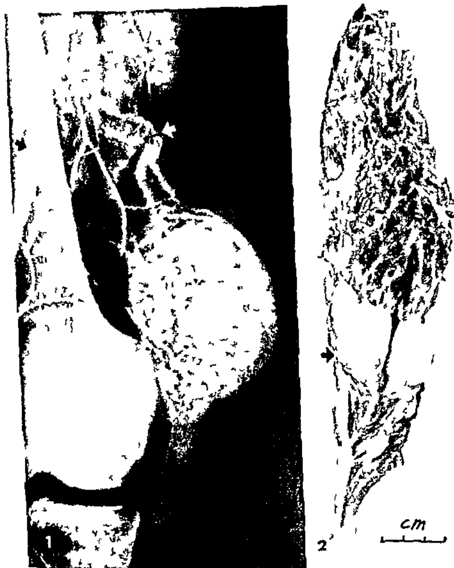
Two years after the excision of the primary tumour iliac and lumbar lymph node metasta es were removed by a retroperitoneal dissection Three months later the patient died with metastatic lesions in the skull the myocardium the peri cardium and the right suprarenal gland

Case 4 A man 60 had noticed a growing mass anteriorly in the left l wer leg for 2 months

On examination a firm tender tumour 5 × 3 × 3 cm was palpated in the an terior muscle compartment of the lower leg 20 cm distal to the knee joint The tumour was free from the skin and the skeleton It moved during isotonic con traction of the tibialis anterior but not during such contraction of the extens r hallucis longus and the extensor digitorum longus

Angiography revealed that the anterior tibial artery was displaced posteriorly in a smooth curve by the tumour but no abnormal vessels were demonstrated nor any arteriovenous shunting

At operation (August 1966) the tumour was found t be locatel t the musculotendinous junction of the tibialis anterior (Fig 2 arrow) This mu cle



Figs 1–2

Fig 1 Cas 3 Femoral angiography demonstrating highly vascular tumour Vein draining the tumour (white arrow) have been filled while contrast mediu still remains in the femoral artery (black arrow) indicating arteriovenou shunting

Fig 2 Case 4 Surgical specimen consisting of tibialis anterior with tumour (ar row) at the musculotendinous junction Note ramifying septa on the cu surfac of the tumour Scale in centimeters

was extirpated together with its fascia Tumour tissue was not exposed during th operation

At follow up examination 2 year and 2 months after the operation there we no signs of recurrence or metastasis

Gross appearance The tumours were roughly spherical or ovoid firm solid rather well demarcated and intimately associated with a tendon in Case 1 presumably (cf Fig 3) one or several tendons passing the posteromedial region of the knee joint (medial head of gastrocnemius semimembranosus semitendinosus and gracilis) in Case 2 the Achilles tendon in Case 3 the tendon of the vastus medialis and in Case 4 the tendon of the tibialis anterior

The cut surface was grey or brownish grey In Case 4 distinct ramifying septa dividing the tumour could be recognized in the cut surface (Fig 2) The largest diameter of the tumour was 7 7 and 5 cm in Cases 2 3 and 4 respectively In Case 1 the tumour itself could not be measured but the circumference of the affected knee was enlarged by 1? cm at the time of biopsy

Histological methods The operative specimens were fixed in 10 per cent formaldehyde solution and embedded in paraffin Five μ thick sections were routinely stained according to the haematoxylin van Gieson method and with haematoxylin and eosin Laidlow s silver impregnation was used for demonstration of reticulin fibres and Weigert s elastin method for studying elastic tissue of tumour vessels Alcian blue and toluidin blue stains were used at pH 4 0 and 0 5 for examination of acid mucopolysaccharides these stainings were done with and without prior treatment of the sections with testicular hyaluronidase (Sigma) The periodic acid Schiff reaction (McManus) was done with and without prior treatment of the sections with diastase (Merck) The tumour cells were examined for cross striation as in rhabdomyoblasts with Masson s trichome staining and Heidenhein s iron haematoxylin staining For demonstration of fat the Sudan Black B method (0 1 per cent solution) was used on paraffin sections and the Scharlach R method on frozen sections (no material available for frozen sections in Cases 1 and 4) Pigment in tumour tissue was analysed with the Prussian blue reaction for iron and reducing properties as in melanin were studied with Masson s and Schmorl s methods

Microscopic appearance (Figs 3–10) All the tumours had a similar strikingly homogenous appearance characterized by round or spindle shaped pale staining epithelioid cells arranged in compact nests and fascicles well defined by septa of fibrous connective tissue The tumour cells exhibited a clear vacuolated or finely granulated cytoplasm with frequently an ill defined border The round or ovoid vesicular nuclei had a large centrally located basophilic deeply staining nucleolus in most of the cells A few multinucleated giant cells with uniform nuclei were demonstrated in Cases 2 3 and 4 Mitoses were scarce or absent The delicate septa of fibrous connective tissue defining the smallest cellular aggregates were continous with coarser fibrous septa transversing the tumour and merging with preformed tendinous tissue (Figs 3 and 8)

Mucoid extracellular material found in Cases 3 and 4 stained positively with Alcian blue and metachromatically with toluidin blue at pH 4 0 but not at pH 0 5 At the former pH the reactions were negative after prior treatment of the sections with testicular hyaluronidase Varying amounts of intracellular diastase sensitive PAS positive material were demonstrated in all 4 tumours suggesting the presence of glycogen Masson s trichrome and Heidenhein s iron haematoxylin

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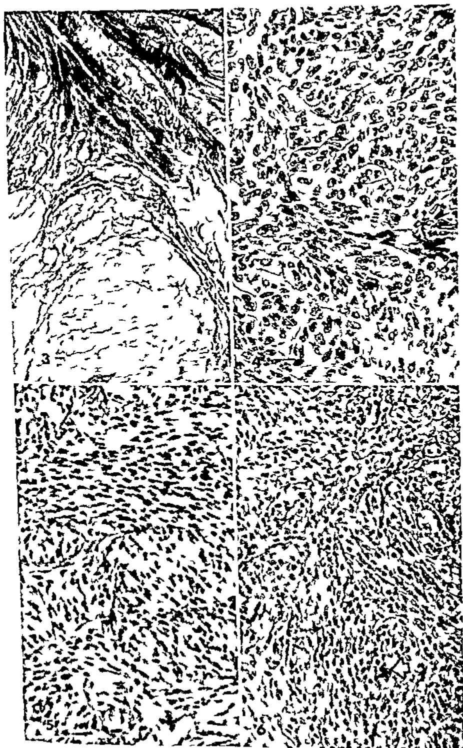
Figs 3 6

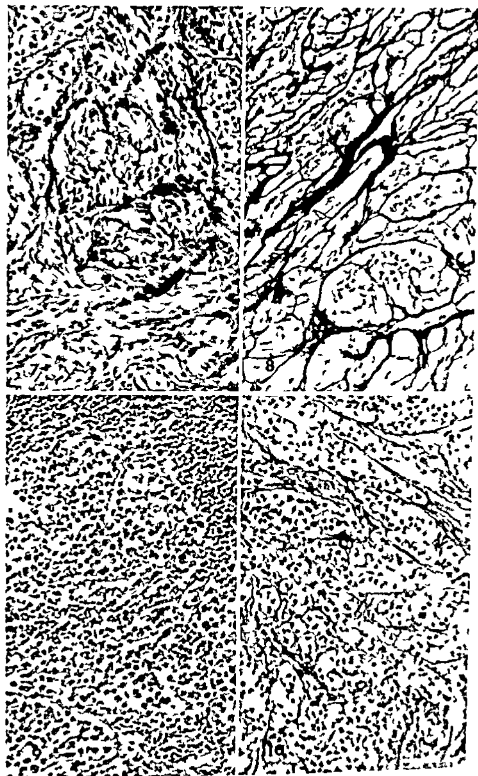
Fig 3 Case 1 Delicate septa of fibrous connective tissue defining the smallest cellular aggregates (cf Fig 4) are continuous with coarser fibrous septa transversing the tumour and merging with tendinous tissue (top) Laidlow stain × 30

Fig 4 Case 1 The tumour is composed of groups of round and spindle shaped sparsely granulated or vacuolated cells with prominent nucleoli *H and E* × 300

Fig 5 Case 2 Section from the tumour showing vacuolated fusiform tumour cells arranged in nests and fascicles H and E × 190

Fig 6 Case 2 Section from another part of the tumour showing a picture similar to that in Fig 4 Arrow indicates giant cell H and E × 190




7
8

stains did not reveal any cross striation of the tumour cells suggesting a myogenic origin. No certain intracellular fat was demonstrated. Pigment was found particularly in the fibrous septa. It gave positive iron reaction indicating the presence of haemosiderin but negative reactions for melanin.

The metastases to the lymph nodes in Cases 2 and 3 displayed a less orderly cellular arrangement (with loss of cellular cohesion), a more granulated and less vacuolated cytoplasm, and a more marked cellular pleomorphism than did the primary tumour (Fig 9). (In Case 1 the lymph node metastases were not available for study.)

Vessels with a diameter of roughly 50–150 microns were chiefly seen in the coarse fibrous septa. Many of them displayed elastic tissue and smooth muscle cells in the wall, but it was not possible to distinguish between afferent and efferent tumour vessels. Few thin walled vessels of capillary type could be recognized within the nests and fascicles of tumour cells in Cases 1, 2 and 4. In Case 3 the tumour was conspicuously vascular with many vessels of capillary or embryonal type in the nests and fascicles of tumour cells.

DISCUSSION

The morphological appearance of the 4 described tumours was characterized by (1) intimate association with a tendon, (2) pale staining, vacuolated of finely granulated cells with prominent nucleoli, and (3) cellular arrangements in nests and fascicles well defined by septa of fibrous connective tissue. This morphological appearance agrees closely with that of clear cell sarcoma of tendons and aponeuroses as described by *Enzinger*.

Earlier this tumour has been interpreted in several instances as synovial sarcoma and this was in fact the diagnosis in Cases 1 and 2 on the histological examination. However, the above mentioned characteristic morphological features and the lack of pseudo acinar structures or other forms of biphasic cellular differentiation help to distinguish clear cell sarcoma from synovial sarcoma. These tumours also differ as to the staining qualities of the secreted mucoid material (*Enzinger*).

In Case 3 the tumour was first interpreted as a fibrosarcoma. However the peculiar cellular features and arrangements and the low degree of desmoplastic activity are not suggestive of a fibrosarcoma (*Enzinger*).

In Case 4 an embryonal or alveolar rhabdomyosarcoma was su

Figs 7–10

Fig 7 Case 3. Vacuolated round and fusiform tumour cells arranged in small nests defined by septa of fibrous connective tissue. H and E. × 190.

Fig 8 Case 3. Delicate ramifying septa of argentaffin fibres enclosing nests of tumour cells. Laidlow stain. × 190.

Fig 9 Case 3. Lymph node metastasis (surgical specimen). Note the dissociation of the tumour cells which show a granulated cytoplasm and pleomorphism. H and E. × 190.

Fig 10 Case 4. Vacuolated mostly round and uniform tumour cells arranged in small nests and fascicles separated by fibrous connective tissue. H and E. × 190.

spected on the initial histological examination However none of the 4 tumours displayed marked acidophilia sarcolemma fibrils or cross striation suggesting rhabdomyoblasts

In some instances of *Enzinger s* series, the tumour had been con fused with a malignant melanoma It is evident that nests and fascicles of spindle shaped cells may have a nevoid feature with some resemblance to that of malignant melanoma of the spindle cell type In no instance however could melanin be demonstrated with different methods neither by *Enzinger* nor by us

The tumours hardly displayed any morphological features of ex traskeletal chondromatous tumours nor could sulphated mucopolys accharides be demonstrated by staining with Alcian blue and toluidine blue at pH 0 5 The results of the mucopolysaccharide stainings com bined with digestion by testicular hyaluronidase indicate the presence of non sulphated mucopolysaccharides such as hyaluronic acid in the mucoid material of the tumours As mucoid material with similar staining properties can be found extracellularly in areas of mucin degeneration in other soft tissue tumours this finding seems to be of slight significance in the diagnosis of clear cell sarcoma of tendons and aponeuroses

Vascular structures were scarce in the tumours studied by *En zinger* One of our tumours however was highly vascular as demon strated both angiographically (Fig 1) and histologically This tumour (Case 3) in contradistinction to the others contained many thin walled vessels of capillary or embryonal type in the nests and fascicles of tumour cells In Case 4 angiography gave the topographic infor mation that the anterior tibial artery had been displaced by the tu mour but no newley *formed vessels were demonstrated*

In all 4 of our patients the tumour was located in the lower ex tremity In *Enzinger s* series 17 out of 21 tumours had this location The mean age of the patients at surgery was 26 years in *Enzinger s* material with only 3 patients being more than 50 years old Three of our patients were between 27 and 39 years old one was 60 Our patients were men In *Enzinger s* series 8 were men and 12 were women

Among 19 patients in *Enzinger s* series on whom follow up infor mation was available 14 had died 6 within 3 years of treatment 8 after a more protracted course lymph node metastases were observed in 7 of the deceased patients Five of the 19 patients with follow up data were alive and well 2–4 years after the last surgical procedure Three of our patients died within 3 years of treatment all with meta stases to lymph nodes The fourth patient is alive and well less than 3 years after excision of the tumour

The 25 instances of clear-cell sarcoma of tendons and aponeuroses hitherto described clearly demonstrate that the tumour is malignant In fact no case of 5 year survival after the last occasion of treatment

has yet been reported It appears that one factor behind the poor result of treatment obtained so far might have been a failure to recognize the ominous nature of the lesion on the first occasion of treatment In 5 patients in *Enzinger's* series amputation was resorted to only when one or several local operations had proved to be inadequate all these patients died In 6 others who died more than one local operation was done suggesting that the initial procedure had not been radical enough When planning an operation with a view to completely removing a clear cell sarcoma of tendons the propensity of the tumour to metastasize to lymph nodes should be taken into consideration Perhaps a higher cure rate can be obtained if the regional lymph nodes are removed along with the tumour preferably in a monoblock procedure

SUMMARY

Four cases of clear cell sarcoma of tendons are presented This is a rare malignant tumour described by *Enzinger* in 1965 Our tumours occurred in men 27 28 39 and 60 years old all tumours were located in the lower extremity and were closely associated with tendinous tissue The 3 younger patients died with multiple metastases within 3 years of treatment all with metastases to lymph nodes The older man is still alive without signs of recurrence or metastasis 2 years and 2 months after excision of the tumour

Differential diagnosis is discussed on the basis of *Enzinger's* and our own morphological observations Angiographic and histological examinations indicate that clear cell sarcoma can be either poorly or highly vascular The propensity of the tumour to metastasize to lymph nodes should be taken into consideration when planning the treatment

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University of Oslo Oslo Norway

CUSHION LIKE INTIMAL LESIONS IN INTRAMYOCARDIAL ARTERIES OF MAN

Their Relation to Age, Sex, Coronary Atherosclerosis and Certain Diseases

By

JØRGEN W HÆREM

Received 13 v 69

In transverse sections of intramyocardial arteries of man cushion like intimal thickenings are frequently seen They are characterized by subendothelial deposits of a structureless material

In a previous autopsy study the morphology of these lesions was examined in order to explore their pathogenesis (6) The most likely explanation of the lesions was that they are the results of haemodynamic trauma against the vessel wall

In the present investigation further information about the pathogenesis of the intimal cushion like lesions is obtained by studying associations between the lesions and age sex coronary atherosclerosis and several diseases present in the autopsy series

MATERIAL AND METHODS

The consecutive autopsy series was the same as in the former study (6) It consisted of 129 males and 80 females from 1 to 96 years of age (Fig 1) One hundred and twenty two men and 84 women were 40 years of age and older the median age of men and women was 65 and 73 years respectively

The autopsies were performed 8 to 34 hours after death A detailed description of the sampling of the myocardium the processing of histological sections and of staining methods has already been given (6)

Microscopical screening with a magnification of 100x was performed in all hearts of one section from the interventricular septum and of one from the lateral wall of the left ventricle In randomly selected cases one section from other parts of the ventricle also was screened All clinical and autopsy data were unknown during the microscopical examination Sections containing recent or old infarcts were not screened

As the cushion like intimal lesions rarely were found in arteries larger than 150 microns or in arterioles smaller than 30 microns in diameter only arteries within these limits were counted They were [illegible] red as having or not having intimal

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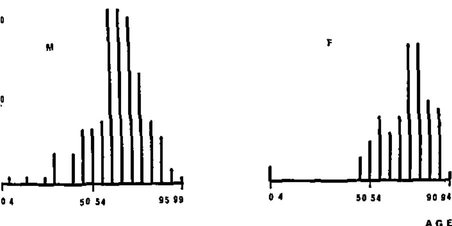


Fig 1
Diagram showing the age distribution of the 1?9 males (M) and the 86 females (F) included in the stud Each column represents one five year group

cushions Fifty arteries were counted in each section if a sufficient number of arteries was present

According to the percentage of myocardial arteries affected the hearts were classified into two groups

1 Ten per cent or less of the arteries affected
2 More than 10 per cent of the arteries affected

Macroscopical examination Total heart weight was noted The heart preparations included the first 2–3 cm of aorta Myocardial necroses or scars larger than one cm in any plane of section were recorded as infarcts The main coronary arteries and their larger branches were opened longitudinally until a luminal diameter of about one mm

According to a subjective estimation of the degree of atherosclerotic stenosis in the coronary arteries the hearts were classified into four groups

1 No stenosis
Stenosis reducing the original lumen by 50 per cent or less in one or more of the three main arteries
3 Stenosis reducing the original lumen by more than 50 per cent in one or more of the main arteries up to complete occlusion in two of the three main arteries
4 In all of the three main arteries complete atherosclerotic occlusion of the lumen

No attempt was made to differentiate atherosclerotic plaques from organized coronary thromb

Statistical methods employed in analysing the results were the χ square method with Yates correction in 2 by 2 contingency tables the χ square test for trend (8) Student s t test and Fisher s exact probability test (8)

RESULTS

The cushion like lesions were observed in one or more of the myocardial arteries in the majority of cases The frequency of affected arteries was about the same in different parts of the same heart



Fig 2
Intramyocardial artery containing a large intimal cushion rich in elastic material (dark grey) The lesion tapers at both sides and the elastic material merges with the internal elastic membrane (arrows) Gomori s aldehyde fuchsin × 710

The morphology of the cushions has been described elsewhere (6) They were elongated spindle shaped protrusions extending along the longitudinal axis of the arteries Predilection sites were at and distal to bifurcations and branching sites of small arteries In cross sections they appeared as cushion like subendothelial deposits tapering at one or both sides and merging with the internal elastic membrane (Fig 2) Disruption and new formation of elastic fibres was observed within the cushions together with modified smooth muscle cells or uncharacteristic rounded cells In some deposits a few mononuclear blood cells were present lipid and rarely material stained as fibrin could be seen

The intimal cushions were not found in any of the nine cases younger than 40 years These cases (7 males and 2 females) are therefore not included in the following analysis

Age and Sex

In groups of increasing age increasing proportions of men had more than 10 per cent of the myocardial arteries affected by cushion like intimal lesions (Table 1) In women the same trend was seen but it did not attain statistical significance at the 5 per cent probability level (Table 2)

The trend was largely unchanged when cases with coronary atherosclerotic stenosis grade 4 were excluded

In men younger than 55 years of age the frequency of arteries

TABLE 1

The Association between Age and the Occurrence of Myocardial Arteries Affected by Cushion Like Lesions in Men

	Total no of cases	Age group		
		40–64 no	65–74 no	75 + no
None to 10 per cent of the arteries affected by intimal lesions	83	38 (79 %)	29 (67 %)	16 (52 %)
More than 10 per cent of the arteries affected by intimal lesions	39	10 (21 %)	14 (33 %)	15 (48 %)
Total	122	48 (100 %)	43 (100 %)	31 (100 %)

χ^2 for the trend that the number of cases with more than 10 per cent of the myocardial arteries affected by cushion like lesions increases by groups of increasing age = 6 57 0 025 > P > 0 01

affected by cushion like lesions did not exceed 10 per cent in any heart Except for one woman of 47 this was also true for women Within each age group the frequency of affected arteries was about the same in both sexes

Coronary Atherosclerotic Stenosis

Cushion like intimal lesions in the myocardial arteries were less frequent in cases with pronounced coronary atherosclerotic stenosis than in cases with no or slight stenosis (Tables 3 and 4) This inverse relationship between lesions of the small and large arteries was statistically significant in both sexes

TABLE 2

The Association between Age and the Occurrence of Myocardial Arteries Affected by Cushion Like Lesions in Women

	Total no of cases	Age group		
		40–64 no	65–74 no	75 + no
None to 10 per cent of the arteries affected by intimal lesions	52	15 (68 %)	18 (72 %)	19 (51 %)
More than 10 per cent of the arteries affected by intimal lesions	32	7 (32 %)	7 (28 %)	18 (49 %)
Total	84	22 (100 %)	25 (100 %)	37 (100 %)

χ^2 for the trend that the number of cases with more than 10 per cent of the myocardial arteries affected by cushion like lesions increases by groups of increasing age = 2 10 0 20 > P > 0 10 NS

TABLE 3

The Association between Coronary Atherosclerotic Stenosis and the Occurrence of Myocardial Arteries Affected by Cushion Like Lesions in Men

	Total no of cases	Degree of coronary atherosclerotic stenosis			
		1	2	3	4
		no	no	no	no
None to 10 per cent of the arteries affected by intimal lesions	83	10 (50 %)	27 (61 %)	21 (70 %)	25 (89 %)
More than 10 per cent of the arteries affected by intimal lesions	39	10 (50 %)	17 (39 %)	9 (30 %)	3 (11 %)
Total	122	20 (100 %)	44 (100 %)	30 (100 %)	28 (100 %)
Median age		65.5	69	65	66.5

χ^2 for the trend that the number of cases with more than 10 per cent of the myocardial arteries affected by cushion like lesions decreases by increasing degrees of coronary atherosclerotic stenosis = 9.44 $P < 0.005$

TABLE 4

The Association between Coronary Atherosclerotic Stenosis and the Occurrence of Myocardial Arteries Affected by Cushion Like Lesions in Women

	Total no of cases	Degree of coronary atherosclerotic stenosis			
		1	2	3	4
		no	no	no	no
None to 10 per cent of the arteries affected by intimal lesions	52	15 (62 %)	11 (47 %)	7 (47 %)	19 (91 %)
More than 10 per cent of the arteries affected by intimal lesion	32	9 (38 %)	13 (53 %)	8 (53 %)	2 (9 %)
Total	84	24 (100 %)	24 (100 %)	15 (100 %)	21 (100 %)
Median age		63	74.5	73	74

χ^2 for the trend that the number of cases with more than 10 per cent of the myocardial arteries affected by cushion like lesions decreases by increasing degrees of coronary atherosclerotic stenosis = 4.53 $0.05 > P > 0.02$

There was no definite change in the degree of atherosclerotic stenosis among men within the age groups studied (Table 5). As regards women, those in the older age groups had more severe atherosclerotic stenosis than those in the younger (Table 6).

Heart Weight

No correlation was found between the prevalence of cushion-like lesions and increasing heart weight in either sex.

No significant correlation of heart weight to age was observed. Exclusion of cases with diseases associated with increased or decreased heart weight[1] did not change this. This was also true when relative heart weight (*i.e.* total heart weight related to body height or body weight) was considered.

In both sexes the largest mean heart weight was observed in the hearts with pronounced degrees of coronary atherosclerotic stenosis.

TABLE 5

The Association between Age and Coronary Atherosclerotic Stenosis in Men

Degree of coronary atherosclerotic stenosis	Total no. of cases	Age group 40–64	65–74	75 +
		no.	no.	no.
1–2	64	24 (50 %)	21 (49 %)	19 (61 %)
3–4	58	24 (50 %)	22 (51 %)	12 (39 %)
Total	122	48 (100 %)	43 (100 %)	31 (100 %)

TABLE 6

The Association between Age and Coronary Atherosclerotic Stenosis in Women

Degree of coronary atherosclerotic stenosis	Total no. of cases	Age group 40–64	65–74	75 +
		no.	no.	no.
1–2	48	20 (91 %)	11 (44 %)	17 (46 %)
3–4	36	2 (9 %)	14 (56 %)	20 (54 %)
Total	84	22 (100 %)	25 (100 %)	37 (100 %)

χ^2 for association between age and degree of coronary atherosclerotic stenosis = 13.8, df = 2, $P < 0.01$

[1] *i.e.* 8 men and 53 women, each with one or more of the following diseases: Old or healing myocardial infarct, myocardial fibrosis and heart failure, valvular heart disease, chronic alcoholism with or without liver cirrhosis, arterial hypertension, chronic renal disease, cerebral or subarachnoidal haemorrhage, ischaemic cerebrovascular disease, chronic obstructive lung disease and malignant neoplasm.

TABLE 7
The Association between Heart Weight and Degree of Coronary Atherosclerotic Stenosis

Degree of coronary atherosclerotic stenosis	No of cases	Mean heart weight (gm)	± SD
Men			
1–2	64	428	130
3–4	58	511	160
Women			
1–2	48	366	121
3–4	36	438	137

t for difference in mean heart weight in men = 3.89 df = 120 $P < 0.001$
t for difference in mean heart weight in women = 2.71 df = 82 $P < 0.01$

(Table 7) When cases with diseases associated with increased or decreased heart weight were excluded this trend was still seen although not quite as distinct and it did not attain statistical significance

Certain Diseases

In the group of patients with *myocardial infarcts of recent origin* only (*i e* < 4 weeks) there was a low frequency of cases with more than 10 per cent of the myocardial arteries affected by cushion like lesions compared with the remaining of the total material of 206 cases (Table 8) The same low prevalence of intimal cushions was found in the group of cases with both recent and old infarcts although it is not statistically significant at the five per cent probability level

In the group of patients with only old infarcts the prevalence of cases with more than 10 per cent of the myocardial arteries affected by intimal cushions was about the same as in the remaining total material The difference between the low prevalence among cases with only recent infarcts and the higher prevalence among cases with only old infarcts attains statistical significance ($P = 0.02$)

When men and women were regarded separately the results in each sex did not differ from those presented above

In *diabetes mellitus* the prevalence of cases with more than 10 per cent of affected myocardial arteries was low but when compared with the prevalence of lesions in the rest of the entire series the difference does not attain the level of five per cent probability (Table 8)

In *senile dementia* 6 out of the 7 cases had more than 10 per cent of affected myocardial arteries (Table 8)

In *cirrhosis of the liver* the corresponding numbers were 5 of 7 cases (Table 8)

TABLE 8

The Association between Certain Diseases and the Occurrence of Myocardial Arteries Affected by Cushion Like Lesions

Disease	No of cases	More than 10 % of the arteries affected No of cases	P for difference from total material §	Athero sclerotic stenosis grade 4 No of cases	Median age
Recent myoc infarct	23	2	<0 05	9	67 5
Recent and old myoc infarct	20	2	NS	14	71 5
Old myoc infarct	21	8	NS	5	67
Sudden coronary death	11	3	NS	7	72
Diabetes mellitus	14	1	NS	6	66 5
Malignant neoplasm	43	17	NS	4	67 5
Senile dementia	7	6	<0 005	2	83
Cirrhosis of liver	7	5	<0 05	0	73
Old rheum valvular dis	10	5	NS	5	78
Accidents acute intox	19	9	NS	0	72
Total material of cases 40 years of age and older	206	61		49	70 5

Some cases have been recorded more than once according to the presence of more than one of the listed diseases Men and Women are grouped together

§ All the P values represent the outcome of χ square tests or Fisher's exact probability test in 2 by 2 tables each disease group against the remaining total material

DISCUSSION

Intimal injury caused by haemodynamic mechanisms is supposed to be of major importance in the pathogenesis of the intimal cushion like lesions (6)

In the present study the frequency of intimal cushions appeared to increase with age and on the other hand severe coronary athero sclerotic stenosis was associated with a low frequency of intimal cush ions These findings do not conflict with a theory of a haemodynamic injury being involved in the pathogenesis of the cushions Firstly if the finding in old rats of a definite increase in susceptibility of coron ary arteries to induced intimal and medial lesions (11) also is valid for man this may imply that haemodynamic trauma is able to induce intimal lesions particularly in old age Secondly as severe arterial stenosis reduce distal blood flow and blood pressure (3 5 9) the haemodynamic mechanisms in the intramyocardial arteries may be modified by the development of severe stenosis in the main epicardial arteries This could be some of the reason for the sparcity of intimal cushions in cases with severe coronary stenosis

If haemodynamic trauma is of importance in the pathogenesis of the cushions a positive relation would possibly exist between the

cushions and high blood pressure. There was no correlation between the frequency of the cushion like lesions and increasing heart weight. However, in order to be able to draw any conclusions regarding heart weight and the occurrence of these lesions, it was also necessary to analyse (a) the relation of age and heart weight and (b) the relation of coronary atherosclerotic stenosis and heart weight. An inverse or positive correlation in (a) or (b) respectively would possibly obscure any connection between the frequency of cushion like lesions and heart weight.

(a) No significant correlation between age and heart weight was observed.

(b) A positive correlation between coronary atherosclerotic stenosis and heart weight was demonstrated. Therefore, a possible correlation between the frequency of cushion like lesions and heart weight could have been obscured by the inverse correlation between lesions and severe coronary atherosclerotic stenosis.

Thus, provided that increased heart weight (more than 500 g in men and more than 450 g in women) reflects hypertension (2), there is no clear evidence that high blood pressure is related to a high frequency of cushion like lesions. On the other hand, such a relationship cannot be excluded.

Considering the various disease groups, it is possible that the low frequency of cushion like lesions in diabetes mellitus is partly related to the lower median age of subjects in this group, partly to the severe degree of atherosclerotic stenosis in the group.

In senile dementia, the high frequency of cushion like lesions may be explained by the old age alone.

In cirrhosis of the liver, severe coronary atherosclerotic stenosis was not present, and the occurrence of many cushion like lesions is consistent with the general tendency of the lesions to be prevalent in cases with little or no stenosis in the large epicardial arteries.

The varying frequency of cases with the higher prevalence of cushion like lesions in the three infarct groups is somewhat difficult to understand on the basis of differences in age and atherosclerosis alone. In the group of patients with only old infarcts, the frequency of cases with coronary atherosclerotic stenosis grade 4 was somewhat lower than in the group with only recent infarcts. However, cases with the higher prevalence of cushion like lesions were much more frequent in the group of old infarcts compared with the group of recent infarcts. As the median age was about the same in the two groups, the possibility may be suggested that the passing through episodes of *myocardial infarcts* perhaps augments the intimal cushions.

Some augmenting effects of myocardial infarcts could possibly be due to factors causing endothelial injury. Such factors could be aggregates of erythrocytes seen in abnormal states (7, 12), increased reactivity of platelets in patients with recent or old myocardial infarcts

(1 10) or possibly the leucocytosis in recent infarction Vasospasm in the myocardial small vessels during infarction causing obstruction to the blood flow (4) could also possibly lead to endothelial injury

The low prevalence of cushion like lesions in the group of cases with both recent and old infarcts does not exclude the possibility of an augmenting effect of myocardial infarcts The frequency of severe atherosclerotic stenosis was high in this group and the general tendency of few cushions in cases with severe atherosclerotic stenosis may counterbalance the possible augmenting effect of an infarct

However these associations do not necessarily indicate a direct causal relationship

SUMMARY

In a previous work haemodynamic mechanisms were supposed to be of importance in the pathogenesis of cushion like thickenings in the intramyocardial arteries

In order to obtain further information about the pathogenesis of these lesions the frequency of the lesions in various conditions and diseases was studied in a consecutive autopsy series of 215 cases The lesions were found in the majority of cases older than 40 years and they were not seen in cases younger than this The prevalence of the lesions increased with age They were inversely related to severe coronary atherosclerotic stenosis The lesions were more frequent in cases with old myocardial infarcts than in those with recent infarcts

It is likely that haemodynamic mechanisms are of importance in the pathogenesis of the intimal cushions and the present results are consistent with this However other factors appearing in myocardial infarction may also be operating

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CHANGES IN SENSITIVITY TO ANGIOTENSIN AND RENIN

Studies on Normal Nephrectomized, Ureterligated Hypoxic Partially Corticectomized or Medullectomized and Renal or Spontaneously Hypertensive Rats

By

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It is wellknown that the sensitivity of rats to angiotensin and renin and other pressor substances is rather different even when normal rats of the same strain age weight and sex are used Such variation in the sensitivity is also found when nephrectomized rats are used for the assays but normal and nephrectomized rats differ in their pattern of response to renin the nephrectomized responding with an increased and prolonged blood pressure rise (for literature see *Page & McCubbin*, 1968) The varying results of studies on this change in sensitivity seem to be due to the fact that many investigators have used different species of experimental animals and different forms of anaesthetics pretreatment of the animals and of doses and purity of the renin preparations

It has been the aim of the present study to obtain further information about the mechanism causing the changed sensitivity to renin in nephrectomized rats For this purpose sensitivity to angiotensin and renin were studied in normal nephrectomized ureterligated hypoxic partially corticectomized or medullectomized and renal or spontaneously hypertensive rats The reason for using these differently pretreated rats was partly that they were used in a previous study on experimentally induced changes in plasma angiotensinogen and plasma renin (*Bing & Poulsen* 1969) and partly that it was thought of interest to compare the changed sensitivity of nephrectomized rats with that of rats with other forms of changed renal morphology and (or) function The study of ureterligated rats should thus allow an estimate of the influence of uraemia the partially corticectomized rats and those partially medullectomized being thought to illustrate the influence of lack of cortical or medullary tissue the study of hypertensive rats could show the influence of the blood pressure level

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MATERIAL AND METHODS

Animals White female rats weighing 180–200 grams from the strain of Wistar from Leo Pharm Trade Co were used for nearly all experiments The rats with spontaneous hypertension belonged to the strain of S A from The Robert Jones and Agnes Hunt Orthopaedic Hospital England[1] The material includes 69 rats eighteen normal seven 22–24 hours nephrectomized eight 22–24 hours ureterligated ten 16–18 hours hypoxic five 22–24 hours partially corticectomized six 22–24 hours partially medullectomized nine renal hypertensive rats studied about 3 months after a clamp was placed on one of the renal arteries and six rats with spontaneous hypertension

The methods for partial *corticectomy* and *medullectomy* partial clamping of the renal artery and hypoxia were those recently described (*Bing & Poulsen* 1969)

The *pressor response* to angiotensin and renin was studied in rats anaesthetised with 20 mg of amytal and pretreated with 5 μg of ergotamine tartrate 1 2 and 2 4 ng of angiotensin research Standard A and 14 mU of Haas Goldblatt Standard (G U) Renin being injected intravenously

RESULTS

1 *The Maximum Increase in Blood Pressure*

In *normal* rats the pressor response to intravenous injection of 2 4 ng of angiotensin was found to vary from about 10 to about 40 mm Hg with a single value of well over 50 mm Hg and the pressor response to 14 mU of Haas Goldblatt renin to follow the variations in responsiveness to angiotensin so that the maximum rise in the individual rat was about the same the response to angiotensin being about 90 per cent of the response to renin (Fig 1) Normal *relation between sensitivity to angiotensin and renin* was also found in the spontaneously hypertensive rats but both in the operated and in the hypoxic rats the maximum increase in blood pressure was markedly higher after injection of renin than after injection of angiotensin (Fig 1 and 2) In renal hypertensive rats the response to angiotensin was somewhat higher than the response to renin The deviation from the normal ratio between the responses to angiotensin and renin was due to changes in both

The sensitivity to angiotensin was markedly decreased in all non normal groups While nearly all normal rats responded to 2 4 ng angiotensin with a more than 20 mm Hg high increase in blood pressure the increase was somewhat lower in *hypoxic* rats (about 10–20 mm Hg) still lower (about 7–20 mm Hg) in *ureterligated nephrectomized partially corticectomized* and *spontaneously hypertensive* rats and very low (5–10 mm Hg) in partially *medullectomized* and in *renal hypertensive* rats

The sensitivity to renin was changed in another way While most of the *nephrectomized* and partially *corticectomised* rats reacted to 14 mU of renin with pressor responses of 30 to well over 50 mm Hg thus being similar to or slightly higher than those found in most *normal* rats (Figs

[1] The author is thankfull to prof *Morten Simonsen* for supplying the S A rats

[2] Both standards were kindly given us from the division of biological standards Medical research council Mill Hill London

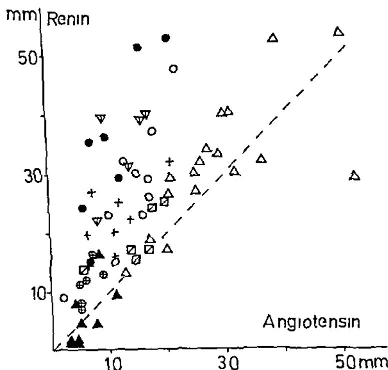


Fig 1

Relation between pressor response in mm Hg to 14 milli Units Haas Goldblatt renin and 2 4 ng of angiotensin in rats

△ normal	▽ partially corticectomized
● nephrectomized	⊕ medulle tomized
+ ureterligated	⊠ spontaneously hypertensive
○ hypoxic	▲ renal hypertensive

The dot—and—dash line includes identi al responses to renin and angiotensin

1 2 and 3) the *hypoxic* rats reacted as the normal rats Contrary to these most *ureterligated* and *spontaneously hypertensive* rats reacted with a relatively low response to renin (16–30 mm Hg) the partially *medullectomized* rats with only 7–17 mm Hg and 9 *renal hypertensive* rats with a mean of 8 mm H_g (range 0–17) 6 of them responding with less than 6 mm Hg

2 *The Duration of the Pressor Response*

The typical short normal response to 2 4 ng of *angiotensin* was found in all groups (Figs 2 and 3) It was further found that cessation of continuous injection of 9 ng of angiotensin per min was followed by the same abrupt fall in blood pressure in nephrectomized and in normal rats

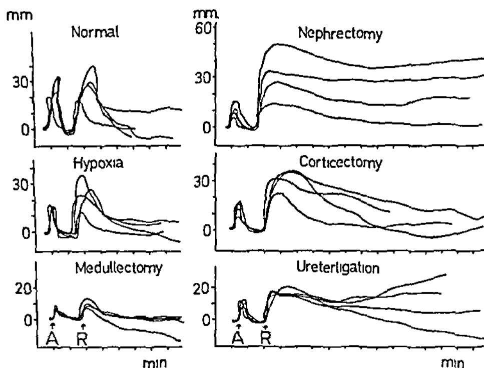


Fig 2

Representative curves showing the pres or response to 2 4 ng of angiotensin (A) and 14 mU of renin (R) in normal 22–24 hours nephrectomized ureterligated partially corticectomized or medullectomized and 17 hours hypoxic rats

The duration of the pressor response and the form of the curve after injection of 14 mGU renin are seen in Figs 2 and 3 Normal rats respond to renin with a rise the velocity of which in most cases is a little slower than that seen after injection of angiotensin followed by a still slower return of the pressure to the starting level the duration of the whole response in most cases being about 3–6 minutes While this level is reached in most cases the response can in some cases one of which is shown in Fig 2 stop at a higher level This is most often found when the blood pressure at the time of the injections is lower than that found immediately after the start of the experiment While the duration of the response to renin is normal in hypoxic and spontaneously hypertensive and often shorter than normal in partially medullectomized and renal hypertensive rats it is prolonged lasting for more than 30 60 minutes in nephrectomized and ureterligated rats being somewhat less prolonged in partially corticectomized rats (Fig 2)

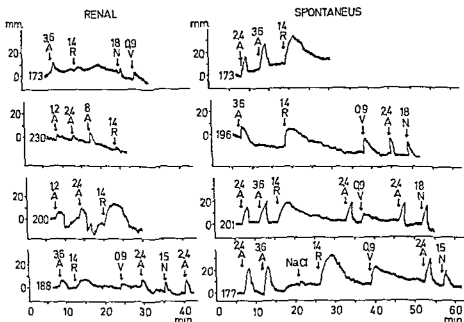


Fig 3

Pressor responses to angiotensin (A) renin (R) vasopressin (V) and noradrenaline (N) in 4 renal hypertensive rats and in 4 rats with spontaneous hypertension The values given are ng for angiotensin in Haas Goldblatt Units for renin mU for vasopressin and ng for noradrenaline The value given in front of each curve indicates the blood pressure in mm Hg

DISCUSSION

1 *Cause of the Sometimes Increased and Always Markedly Prolonged Pressor Response to Renin in Nephrectomized (and Partially Cortiicectomized) Rats*

In most studies on the abnormal response to angiotensin and renin in nephrectomized animals the response to angiotensin has been found to be normal or increased and the response to renin changed being both increased and prolonged The present investigation is so far in agreement with these results as the mean response to renin is higher in the nephrectomized than in the normal rats and the prolonged type of the curve (Fig 2) is quite the same as that previously found But the maximum increase in response to renin in nephrectomized rats is markedly smaller than that reported in the literature and the decrease in response to angiotensin has apparently not been observed in previous studies The reason for these differences is probably to be found in differences in animals anaesthesia pretreatment and renin preparations and doses used in studies like the present

The mechanism involved in the changed response to renin in nephrectomized animals is unknown Many of the primarily possible cau-

ses seem to be excluded (see *Page & McCubbin* 1968) Some of the remaining possibilities will be discussed here

1 The changed response to renin in nephrectomized animals seems not to be due to their increased *plasma angiotensinogen*, as *Carretero & Gross* (1967 b) found the changed response but only slightly increased angiotensinogen in rats tested two hours after nephrectomy The present finding of normal or shortened duration of the renin pressor curves in hypoxic and in partially medullectomized rats in defiance of their markedly increased plasma angiotensinogen (*Bing & Poulsen* 1969) is in agreement with this conclusion

2 According to recent studies by *Sen et al* (1968) the kidneys contain a *phospholipid* which both reduces the blood pressure of renal hypertensive rats and inhibits the pressor response to renin in normal as well as in nephrectomized rats This finding opens up the possibility that removal of this renal phospholipid by nephrectomy is the cause of the changed pressor response The location of the factor inside the kidney has not been determined but other vasodepressive renal factors have been shown to be located to the medulla The present finding of about the same changes in pressor response to renin in partially corticectomized and nephrectomized rats and exactly the opposite changes in partially medullectomized rats shows that if lack of this factor should be the cause of the changed response it must be located to the cortex

3 *Bunag et al* (1968) found that the enhancement of the maximum pressor response in nephrectomized dogs was inhibited if they were pretreated with crude kidney extract hog renin or angiotensin and they therefore believe that the increased pressor response is due to the *depletion of endogenous renin* caused by the nephrectomy Their study does not allow any evaluation of the influence of the treatment with renin or angiotensin on the prolongation of the pressor response which is found after nephrectomy As ureterligated rats have been found to have much higher plasma renin than nephrectomized (and corticectomized) rats (*Bing & Poulsen* 1969) the present finding that the prolongation of the pressor response to renin is quite similar in ureterligated and in nephrectomized rats speaks against the hypothesis that the prolonged response to renin is due to depletion of endogenous renin

4 In a previous study it was found that *uraemia* might perhaps be the cause of the increased angiotensinogen concentration in nephrectomized and ureterligated rats (*Bing & Poulsen* 1969) The present finding about the same prolonged response to renin in nephrectomized partially corticectomized and ureterligated rats could speak for a causal relationship between uraemia and prolonged response to renin The finding of just the same degree of uraemia in partially medullectomized and in partially corticectomized rats both of which have plasma creatinine concentrations of 3 to 4 mg per cent however speaks against this

explanation as the medullectomized rats do not react with a prolonged but on the contrary with a shortened response to renin

5 The typical prolongation of the response to renin in nephrectomized animals cannot be explained by a prolonged persistence of the injected renin in the blood of nephrectomized animals as *Schaechtelin et al* (1964) found the same half life for renin in plasma of normal and nephrectomized rats They therefore assumed that an *accumulation of renin in the arterioles* might be responsible for the prolonged response This hypothesis was supported in studies showing that anti angiotensin injected in doses which neutralizes renin in normal rats and angiotensin both in normal and nephrectomized rats will only diminish the maximum increase and will not change the prolonged form of the response to renin in nephrectomized rats (*Bing & Poulsen* (1968) A direct proof of the correctness of this hypothesis by determinations of the arteriolar renin content after renin injection into normal and nephrectomized animals however is lacking

2 *Cause(s) of the Low but Markedly Prolonged Response to Renin in Ureterligated Rats*

The cause(s) of the changed response to angiotensin and renin in ureterligated rats (Fig 2) is (are) still unknown It is possible that the decrease in maximum response is caused by the same unknown mechanism which causes the still lower response in partially medullectomized and renal hypertensive rats The cause of the prolongation of the response to renin may be the same as that which causes the prolonged response in nephrectomized and partially corticectomized rats

3 *Cause(s) of the Low response to Angiotensin and Low and Short Response to Renin in Partially Medullectomized and Renal Hypertensive Rats*

The cause(s) of the changed response to angiotensin and renin in partially medullectomized and renal hypertensive rats (Figs 2 and 3) is (are) unknown The present study shows that the change in response to renin so far should not be believed to be due to an inhibitor of the reaction between renin and its substrate angiotensinogen as the response to angiotensin is equally or still more reduced The very low response to angiotensin and renin in renal hypertensive rats seems not to be due to the high level of the blood pressure as spontaneously hypertensive rats with the same blood pressure level have only moderately reduced ... e due to angiotensin—and renin ta ... o noradrenaline and vasopressin we-

SUMMARY

1 The *sensitivity to angiotensin* was found to be somewhat decreased in hypoxic rats more decreased in ureterligated nephrectomized partially corticectomized and spontaneously hypertensive rats and very low in partially medullectomized and in renal hypertensive rats

2 The *sensitivity to renin* was as regards the *maximum increase* normal or increased in nephrectomized and partially corticectomized rats normal in hypoxic rats somewhat decreased in ureterligated and spontaneously hypertensive rats markedly decreased in partially medullectomized rats and still more decreased in renal hypertensive rats in which the sensitivity to angiotensin noradrenaline and vasopressin was about equally low The *duration* of the pressor response was about equally markedly prolonged in nephrectomized and ureter ligated rats and also markedly but somewhat less prolonged in partially corticectomized rats

3 The most recent hypotheses dealing with the explanation of the *mechanism of the changed response to renin in nephrectomized rats* are discussed in relation to the results of the present study It is concluded that the prolonged response to renin in nephrectomized rats is not due either to increased plasma angiotensinogen or a medullary inhibitor of the renin angiotensinogen reaction nor to depletion of endogenous renin or to uraemia

4 The mechanism of the *changed response to angiotensin and renin* (decreased maximum response to both and prolonged response to renin) in *ureterligated* rats is unknown The decreased response to both angiotensin and renin in *renal hypertensive rats* and in *partially medullectomized* rats seems not to be due to an inhibitor of the reaction between renin and angiotensinogen nor to the blood pressure level or to renintachyphylaxis

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HISTORADIOGRAPHIC STUDY OF EXFOLIATED CELLS OF THE ORAL MUCOSA

A Method for Comparison of Historadiograms and Papanicolaou Staining

By

E DABELSTEEN and I P CLAUSEN

Received 25 vi 69

Investigations of the degree of keratinization of exfoliated cells of the oral mucosa have mainly been based on smears stained according to the method described by Papanicolaou (1942) Most of these investigations have been reviewed by *Camilleri & Lange* (1966)

Although the reliability and usefulness of the smear technique as a means of detecting malignances and premalignances has been questioned among others by *Gorlin* (1965) and *Chandler* (1966) the value of the Papanicolaou staining technique for estimating degree of keratinization of squamous epithelial cells is generally trusted This confidence is reflected by the great amount of published studies based on this method

A few authors however *Silverman Beck & Farber* (1958) *Trott* (1962) *Trott & Banoczy* (1962) and *Waller* (1962) have expressed their doubt as to the value of the Papanicolaou staining for evaluating the degree of keratinization of exfoliated cells

An incontestable disadvantage of the Papanicolaou method is its unsuitability for quantitative studies *Onisi & Kesuge* (1963) overcame this difficulty by using the crystal violet and Bismarck brown of a Gram stain Dyes in specimens each containing numerous cells were dissolved in acetone and analyzed by means of an electrophotometer This method however does not allow an estimation of the degree of keratinization of the single cell and the factors influencing the staining reactions are not well understood

Historadiographic studies of skin and oral mucous membrane have shown a very considerable difference in X ray absorption—and hence in dry weight—between the keratinized and non keratinized cell layers of the squamous cell epithelium *Moberger & Engstrom* (1954) *Lindstrom & Moberger* (1955) and *Clausen* (1969) *Muller Sandritter &*

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Schwaiger (1959) developed a method for historadiographic determination of dry weight without use of a reference system using a sealed-off X ray tube *Combée, Houtman & Recourt* (1955) *Müller et al* (1959) showed that this method could be used for quantitative determination of the dry weight of exfoliated squamous epithelial cells of the oral mucous membranes with a mean error of reproducibility of about 10 per cent

The usefulness of the historadiographic method of studying exfoliated cells and the conspicuous difference in dry weight between keratinized and non keratinized cell layers made the authors believe that the historadiographic method could be used to study exfoliated cells of the oral mucosa from patients with disturbances of keratinization *It was also believed that the method could be used to estimate the* reliability of the Papanicolaou technique as an indicator of degree of keratinization

Therefore the purpose of the present investigation has been to work out a method which allows a historadiographic examination and a Papanicolaou staining as well of the same exfoliated cell

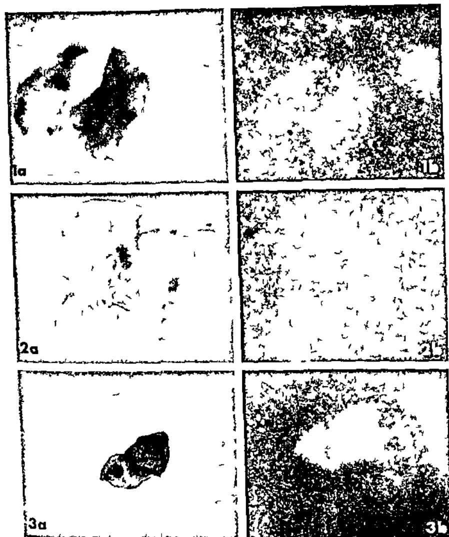
MATERIAL AND METHOD

The studied material consisted of exfoliated cells from the oral mucosa from clinically normal cheeks and hard palates of adult men The scrapings were made with a wooden cotton covered stick moistened in physiologic saline solution They were taken from the area just anterior to the papilla salivalis superior and from the hard palate In order to obtain unkeratinized and keratinized cells on the same historadiogram cells from cheek and palate of the same person in some cases were mixed in the same specimen The sticks were immediately rolled directly onto the emulsion side of an Agfa Gevaert 10E56 film and fixed for 30 minutes in a solution of 96 per cent alcohol and were then air dried.

The films were exposed in a Philips CMR 5 contact microradiograph with equipment very similar to that used by *Müller et al* (1959) For a detailed description of the equipment see *Clausen & Dahlin* (1969) The exposures were made for 40 minutes in continuously evacuated exposure chamber The voltage across the X ray tube was 1 5 kV which with the equipment used gives a radiation of the specimen with polychromatic X rays ranging from a wave length of 8 2 Å to 13 Å The maximum intensity was about 12 5 Å After exposure the film was developed for 5 minutes in Gevaert s developer GP 201 diluted 1 4 with distilled water and then fixed and rinsed thoroughly Thereafter the film with exfoliated cells was stained according to Papanicolaou s technique (*Clayden* 1962) For the purpose of identification of the cells and for comparison of the colour and the radiopacity of the cells all the radiograms were mounted in water between slide and cover glass and microphotographed on Agfa CK 20 colour film Under a drop of water and observed through the low power of a microscope the cells were then removed one by one *from the film by means of a nylon hair* and the historadiograms were dried and mounted in Eukitt® between histologic slides and cover glasses the emulsion side facing the cover glass The historadiograms were examined under a microscope and compared with the colourslides of the corresponding cells

RESULTS

The method is circumstantial and time consuming Although it implies many possibilities of making artifacts *it was successful in nearly all* cases Only in 5 instances was the photographic emulsion damaged



Figs 1–3

Fig 1 a + b The lowest radiopacity is seen in the blue cell there is a higher radio pacity in the red cell and the highest radiopacity is seen in the yellow cell This seems to indicate an increasing dry weight with increasing degree of keratinization and a good accordance between the latter and the staining reaction Note in the cell to the left that the nucleus appears more radiopaque than the surrounding cytoplasm × 300

Fig 2 a + b A faintly and a strongly stained blue cell Note that the strongly stained cell shows a considerably higher radiopacity than the faintly stained cell of the same colour × 300

Fig 3 a + b Two small cells with pyknotic nuclei Both show a high radiopacity of nearly equal intensity in spite of an entirely different staining reaction Note the shrinkage of the cells (a) as it results from the staining it does not influence the radiogram × 300 The relation between colour and radiopacity shown in Fig 2 and 3 possibly indicates a disagreement between staining reaction and degree of keratinization

during the procedure. The Papanicolaou stained cells were categorized according to the colour of the cytoplasm into yellow (keratinized), red (partially keratinized) and blue (non keratinized) types.

Examination of about 17 stained scrapings and the corresponding historadiograms showed that blue cells generally had a less radio-opaque cytoplasm than the red cells (Fig. 1 A + B). However, besides this correlation between the radiopacity and the quality of the colour of the cell, there seemed to be a correlation between the intensity of the colour of the cell and the degree of its radiopacity in such a way that heavily stained blue, red or yellow cells or parts of these were more radiopaque than lightly stained areas (Fig. 2 A + B). Although these were the most common findings, cells could be found with different colours and with a very similar radiopacity (Fig. 3 A + B). The nuclei of the cells were more radiopaque than even the most radiopaque areas of the surrounding cytoplasm independent on the colour of the cytoplasm (Fig. 1 A + B).

DISCUSSION

As shown by *Engström & Lindström* (1949, 1950) and by *Lindström* (1955) ultrasoft X-rays can be used for the determination of the dry weight of cells in deparaffinized sections and in frozen dried sections of soft tissue provided that they are basically built up of carbon, oxygen and nitrogen. In the wave length region used, 8–13 Å, the mass absorption coefficients of high atomic elements are greatly reduced and in oral mucosa high atomic elements are present in very small concentrations as compared with the carbon, oxygen and nitrogen of the proteins. Only sulphur might be suspected to give a stronger X-ray absorption than to its mass. According to *Moberger & Engström* (1954) it can be calculated, however, that even so high a sulphur concentration as 10 per cent does not disturb the dry weight determination. Furthermore, recent studies quoted by *Silverman* (1967) confirm that epidermal keratin differs from some other animal keratins in that it is a very low sulphur containing protein. The relative radiopacity of the cell structures in historadiograms hence reflects the relative distribution of dry weight in the cells.

The technique introduced by *Muller et al.* (1959) made it possible to use the relative simple Philips CMR 5 microradiograph for quantitative studies without a reference system and demonstrated the possibility of determining the dry weight of, among other things, exfoliated cells of the oral mucosa. The present authors have improved the technique further by applying the cells directly onto the emulsion of the film, thus avoiding the 7 µm thick Mylar film formerly used, which has a much too high absorption, a drawback of which *Muller et al.* (1959) (p. 430) were very well aware. The direct application gives the historadiograms a far better sharpness.

As the purpose of the present study was to work out a method to compare the historadiogram and the Papanicolaou staining of the very same cell it was necessary to stain the cells while they were still on the radiogram if identification of cells should not be made unreasonably difficult. In this was a faint staining of the emulsion cannot be avoided. It is important that the Papanicolaou staining is performed after exposure development and fixation of the historadiogram in order to avoid the influence of absorption of X rays by the dyes.

The present method offers considerable advantages compared with the quantitative estimation of the staining of exfoliated cells made by *Onisi & Kosuge* (1963) the historadiographic technique being easier to quantitate and reproduce than the staining technique and because the estimation can be done on the single cell.

Most authors agree that differences in colours of the Papanicolaou stained cells reflect differences in keratinization. If this assumption is correct one should expect blue cells to have a low radiopacity and hence a low dry weight. The radiopacity is supposed to increase in red and yellow cells. In most cases this is what has actually been found. Nevertheless in quite a few cases the degree of radiopacity seemed to be related to the intensity of the staining and not to the colour per se. This correlation might be caused by differences in thicknesses of the cells or it might indicate that the Papanicolaou technique is unreliable as a method of estimating degree of keratinization of exfoliated cells of the oral cavity. To find out which is the case it will be necessary to make cytophotometric measurements of the historadiograms and the stained cells and measurements of the thickness of the cells and then correlate the findings.

On historadiograms of human oral mucous membranes (*Moberger & Engström* 1954 *Clausen* 1969) the nuclei of the epithelial cells present themselves as relatively radiolucent areas with exception of the nuclei of the parakeratotic layer when they consist of intensely compressed cells. On the historadiograms of the exfoliated cells the nuclei when present are invariably radiopaque. This might be explained by the fact that although the nuclei have a lower dry weight than the surrounding cytoplasm and will thus appear relatively radiolucent on tissue section they will appear relatively radiopaque in the exfoliated cells because of the fried egg form of these cells. In spite of the relatively lower absorption of the nucleoproteins compared with that of the plasmaproteins the differences in thickness of the nucleus and the cytoplasm make the former appear the more radiopaque.

SUMMARY

A method is presented which allows the examination of historadiograms and Papanicolaou staining of the same exfoliated cells of the oral cavity. The historadiograms have been made in a Philips CMR 5

contact microradiograph under standardized conditions The radiation consisted of polychromatic X rays ranging in wave length from 8 2 Å to 13 Å The exfoliated cells were placed directly onto the photographic emulsion and the Papanicolaou staining was performed while the cells were still on the film Examination of the historadiograms and the stained cells suggested that the Papanicolaou technique is possibly not a completely reliable method of estimating exactly the degree of keratinization of exfoliated cells This problem however can only be solved on the basis of a quantitative photometric examination of the historadiograms prepared by means of the technique described comparing the results with the thicknesses and staining reactions of the corresponding cells

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ORAL POLYETHYLENE GLYCOL'S INHIBITORY EFFECT ON THE SUBCUTANEOUS GROWTH OF EHRLICH'S CARCINOMA IN MICE, AND ON THE LOCAL INFLAMMATORY RESPONSE TO THE TUMOUR

By

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Polyethylene glycol is a long chain polymer which can potentiate the haemolytic (*McVicar* 1949) and the oncolytic (*Hartveit* 1967) activity of complement It has previously been reported (*Hartveit* 1969) that intraperitoneal treatment with polyethylene glycol 4000 has an inhibitory effect on the subtaneous growth of Ehrlich s carcinoma in female mice of our closed colony It was subsequently remarked that a 20 per cent solution of polyethylene glycol 4000 administered *ad lib* in the drinking water had a similar inhibitory effect In a preliminary experiment 12 female mice given this treatment showed a 48 per cent reduction in subtaneous tumour growth 9 days after transplantation compared to 12 untreated controls ($0\ 001 > P$)

The mechanism underlying this growth inhibition has not been studied previously The experiment was therefore repeated (Exp 1) and extended to include male mice (Exp 2) and histological studies Oral treatment with polyethylene glycol 4000 was accompanied by profuse watery diarrhoea and marked weight loss A further control study was therefore included (Exp 3) in which the mice were given glycerol *per os in an attempt to evaluate the part played by dehydration per se*

In the course of the experiments it became apparent that the inflammatory response to the tumour was a determining factor in its growth This is in keeping with Jones observation (1914) that the presence of a non specific irritant may enhance tumour growth and *Wheatley's* (1963) comparable findings with ascitic growth of this tumour The importance of new connective tissue proliferation has been stressed

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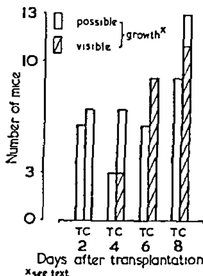


Fig 2
Tumour growth in mice treated with polyethylene glycol (T) and in untreated controls (C) related to days after transplantation

Spleen Weight

At 8 days after transplantation the mean spleen weight ± S D in the polyethylene glycol treated mice was 44 2 ± 25 6 mg while that the controls was 145 3 ± 62 4 mg This difference is statistically significant *(0 001 > P)*

There was no significant correlation between tumour size and final body weight or between body weight and spleen weight at 8 days in either group

Experiment 2 (20 per cent and 10 per cent polyethylene glycol in male mice) The *body weight* of these male mice is also shown in Fig 1 The standard deviation from the means was between 1 4 and 2 9 g The weight of the mice treated with 20 per cent polyethylene glycol fell steadily at about the same rate as that of the females in experiment 1 the weight loss at 8 days being 26 per cent Treatment with 10 per cent polyethylene glycol gave little change in weight which remained similar to that in the controls The difference in weight at 8 days in the mice treated with 20 per cent polyethylene glycol and the un treated controls is statistically significant *(0 001 > P)*

Tumour growth following 20 per cent polyethylene glycol was similar to that recorded in the females in Exp 1 By 8 days possible growth was present in only 4 of the treated mice while growth was visible in 9 and possible in 3 of the untreated controls Following a 10 per cent solution of polyethylene glycol growth was similar to that in the controls

It is of note that the amount of polyethylene glycol taken by the

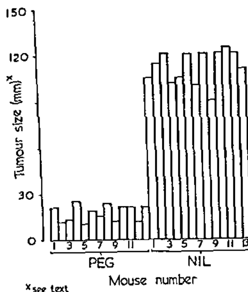


Fig 3
Tumour size at 8 days in mice treated with polyethylene glycol (PEG) compared to that in untreated controls (NIL)

mice given the 10 per cent solution was probably much less than half that taken by those given the 20 per cent solution as the bottles for the latter had to be filled twice as often as the others

Tumour si e as judged from the sum of the two diameters at 8 days was significantly reduced in the mice treated with 20 per cent polyethylene glycol compared to that in the controls (29 1 ± 5 2 mm and 36 7 ± 6 0 mm resp *0 01 > P > 0 001*) Following 10 per cent polyethylene glycol growth was similar to that in the controls The tumour depth varied from 1–2 mm in those given 20 per cent polyethylene glycol and from 2–4 mm in the others Judged from the sum of the diameters multiplied by the depth the group given 20 per cent polyethylene glycol showed a mean reduction in tumour growth of 48 per cent compared to the untreated controls

Spleen weight Following 20 per cent *polyethylene glycol the* mean weight ± S D was 59 2 ± 28 6 mg following 10 per cent the weight was 151 3 ± 74 0 mg and 153 2 ± 69 8 mg in the controls The difference in spleen weight between those given 20 per cent polyethylene glycol and the untreated controls is statistically significant (*0 001 > P*)

The relationships between final body weight and spleen weight and between tumour size and body weight were also investigated There was no significant correlation between any of these factors except for the tumour size and body weight in the group treated with 20 per cent polyethylene glycol where the positive correlation was just statistically

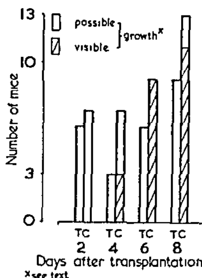


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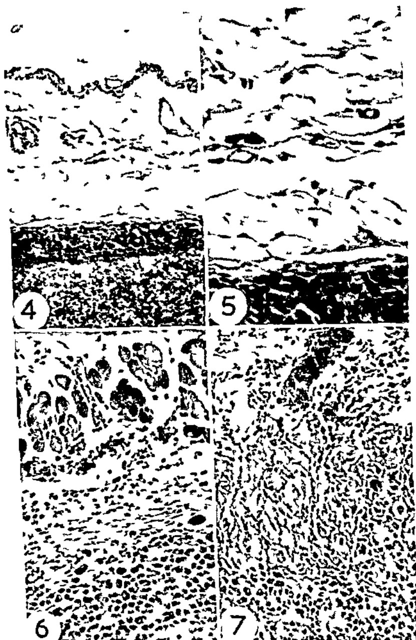
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It is of note that the amount of polyethylene glycol taken by the



Figs 4–7

Fig 4 Eight day tumour transplant in polyethylene glycol treated mouse Note marked lack of proliferation and infiltration (HE × 150)

Fig 5 As Fig 4 Note lack of inflammatory reaction and sharp demarkation between tumour cells (below) and normal tissues (HE × 370)

Fig 6 Eight day tumour transplant in untreated control mouse Tumour growth below panniculus carnosus Note Acute cellular inflammatory exudate Irregular band of tumour cells across lower part of figure with necrotic tumour bottom right. (HE × 150)

Fig 7 As Fig 6 Tumour infiltrating muscle layer Note cellular inflammatory exudate and dilated blood vessels in subcutaneous tissues at top of figure (HE × 150)

The surrounding tissues showed little sign of reaction (Fig 5) The blood vessels appeared to be unaffected and no cellular infiltration was present The line of demarkation between tumour tissue and the muscle layer was sharp Few mitoses were seen

The above histological picture was in distinct contrast to that seen in *the control mice at 8 days* which showed marked infiltrative growth and frequent mitoses Where growth was still confined to areas below the panniculus carnosus (Fig 6) its underlying blood vessels which were not prominent in the treated mice were dilated and filled with blood This hyperaemia was accompanied by exudate formation in the space between the muscle layer and the tumour transplant The exudate showed the beginnings of organization with a thread like matrix interspersed with fibroblasts and infiltrated with granulocytes The tumour cells on the surface of the transplant were irregularly arranged and showed frequent mitoses while those deeper in the transplant were necrotic There was no sharp line of demarkation between transplant and normal tissues Instead this area was filled by exudate as described above in which the actively dividing tumour cells mingled

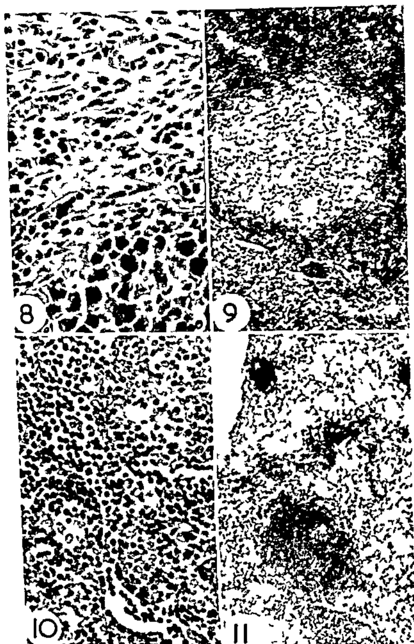
In other areas the inflammatory exudate containing tumour cells was present throughout the muscle layer (Fig 7) In other words the tumour had infiltrated the muscle layer The inflammatory process was also marked in the overlying dermis with dilated blood vessels oedema granulocyte infiltration and scattered tumour cells

In these tumours that had infiltrated the panniculus carnosus the exudate under the muscle layer showed further organization (Fig 8) and now presented a picture of young granulation tissue with fibroblasts and newly formed thin walled blood vessels While these vessels were dilated and blood filled granulocyte infiltration was less marked than in the exudates The tumour cells abutting on to this granulation tissue showed many mitoses In mice treated with *10 per cent polyethylene glycol* and those with *20 per cent glycerol* the histological picture was as in the untreated controls

Lymph Nodes

In the tumour bearing mice treated with *20 per cent polyethylene glycol* the lymph nodes were enlarged at 8 days Follicles were prominent and contained well developed germinal centres with many tingible bodies (Fig 9) The subcapsular sinus was almost completely obliterated being compromised by the diffuse masses of lymphocytes in the outer areas of the follicles Paracortical proliferation was also marked While the cells lining the medullary sinuses were not prominent the sinuses themselves were dilated and tightly packed with mainly small lymphocytes (Fig 10)

The lymph nodes in the untreated tumour bearing controls were also enlarged In contrast to the mice treated with 20 per cent poly



Figs 8–11

Fig 8 As Fig 7 Organized exudate adjacent to tumour cells Note frequent mitoses in neighbouring tumour cells (HE × 370)

Fig 9 Lymph node from mouse with eight day tumour transplant treated with polyethylene glycol Note enlarged follicle with prominent germinal centre and also paracortical proliferation (HE × 150)

Fig 10 As Fig 9 Note masses of small lymphocytes in sinuses (HE × 370)

Fig 11 Spleen from mouse with 8 day tumour transplant treated with polyethylene glycol Note small inactive follicles and lack of cellularity of the pulp (HE × 150)

ethylene glycol these nodes showed only moderate proliferation of the follicles with smaller germinal centres and few tingible bodies Para cortical proliferation was not in evidence The sinuses were dilated lined with rather plump pale staining cells and contained but few lymphocytes

The histological picture in the lymph nodes of tumour bearing mice treated with 10 per cent polyethylene glycol and those treated with 20 per cent glycerol was similar to that in the untreated tumour bearing mice

Spleen

The spleen in mice treated with *20 per cent polyethylene glycol* was atrophic and remarkable histologically for its lack of cellularity The follicles were small and lacked germinal centres The white pulp was represented by a few layers of lymphocytes around the branches of the splenic vessels The red pulp contained few megakaryocytes little extra medullary haematopoiesis and extended right up to the splenic capsule (Fig 11) These findings contrast markedly to that in *untreated non tumour bearing mice* in which follicles are well developed and on occasion contain germinal centres In addition there is usually more white pulp than red and it extends right up to the splenic capsule In *untreated tumour bearing mice* germinal centres are prominent in enlarged follicles The white pulp is extremely cellular and extends to form a band just under the splenic capsule The red pulp is congested and extramedullary haematopoiesis marked at times

In mice treated with 10 per cent polyethylene glycol and those given 20 per cent glycerol the histological findings in the spleen were similar to those described in the untreated tumour bearing mice although the amount of white pulp was reduced in the smaller spleens in both groups

DISCUSSION

The present experiments confirm that oral treatment with polyethylene glycol 4000 will give a significant reduction in subcutaneous tumour growth in both male and female mice As it was previously thought that this reduction in tumour growth might well be due to dehydration per se *i e* that the reduction in tumour size might be merely a reflection of general weight reduction studies were carried out on mice dehydrated with oral glycerol The findings in these mice in which there was a statistically significant positive correlation between tumour size and the degree of dehydration in the treated group as judged from the final body weight supports this hypothesis However although the distribution of both tumour size and spleen weight within the

My thanks are due to Dr B Halleraker of this Institute for suggesting the use of glycerol

glycerol treated group could be shown to be related to the final body weight i e to be influenced by dehydration the extent of tumour growth in the group as a whole was not effected by treatment as the mean tumour size did not differ significantly from that in the untreated controls

In the mice treated with 20 per cent oral polyethylene glycol and also in the untreated mice in all experiments no such correlations were found within the groups That is to say no relationship could be demonstrated between tumour growth or spleen weight and the degree of hydration of the host A possible exception was the group of male mice treated with 20 per cent polyethylene glycol in which the positive correlation between tumour growth and body weight was just statistically significant However this correlation was obtained from the sum of the two diameters of the tumours No correlation was found to tumour depth As tumour depth is a measurement that is likely to be influenced by the turgidity of the tissues correlation with this factor and possibly spleen weight too as was present in the glycerol treated mice should probably be required before relationship to dehydration can be imputed with any certainty

While dehydration as evidenced by diarrhoea and weight loss was certainly present in the polyethylene glycol treated mice it thus seems that it is unlikely to be the only or even the main factor responsible for the reduction in tumour growth seen in these mice although reduction in tumour growth did not occur in its absence

Reduction in tumour growth was only achieved with 20 per cent polyethylene glycol so high dosage appears essential Reduction in tumour growth was also regularly accompanied by reduction in spleen weight The histological picture of splenic atrophy in these mice is in striking contrast to the cellularity usually seen in the spleens of tumour bearing mice whether dehydrated by glycerol or untreated This marked splenic atrophy is reminiscent of that seen under general conditions of stress However the lymph nodes then show a similar atrophic picture In contrast the lymph nodes in these mice were enlarged and the enlargement was due to cellular proliferation both in the follicles which showed hyperactive germinal centres and paracortically Although the lymph nodes in tumour bearing mice not treated with polyethylene glycol were also enlarged lymphocyte proliferation was by no means so marked particularly paracortically Further in the untreated tumour bearing mice the findings in the spleen and nodes were comparable The dissociation between the findings in the spleen and lymph nodes in polyethylene glycol treated mice is therefore unusual and needs further investigation before its aetiology can be discussed with profit

The main finding reported in this paper concerns the difference in the histology of the subcutaneous tumours in polyethylene glycol treated mice and their untreated controls which has been described

in detail From these findings it is clear that subcutaneous growth of this tumour is associated with the presence of an inflammatory reaction in the surrounding normal tissues In the absence of such a reaction infiltrative tumour growth did not occur Expansive growth *i e* the layering of one cell layer on the other did occur This gave a pattern of rows of cells parallel to the surface of the transplant It seemed that a layer of tumour cells about 10 cells deep could obtain sufficient oxygenation by diffusion for survival but proliferation was not active Deeper than this the transplant showed a uniform necrosis in the absence of bleeding or any other reactive changes This is hardly surprising as there was no sign of vascularization of the transplants at all

The surface of the transplant in these polyethylene glycol treated tumours was sharply demarkated from the normal tissues which appeared to be completely unaffected by its presence there being no oedema inflammatory cell infiltration or changes in the blood vessels The tumour cells on the surface of the transplant are thus surviving but the tumour seems unable to establish any stromal reaction While the cells themselves have previously proven their ability to grow in a malignant fashion and do so again in the controls they do not behave in that way under the present circumstances The initial transient swelling at the injection site in the polyethylene glycol treated females suggests that an inflammatory reaction may have occurred in these mice too before treatment took effect

This failure to establish infiltrative growth could be due to a direct effect of polyethylene glycol on the tumour cells The possibility can not be ruled out at present but it is unlikely as this substance is not toxic to tumour cells in short term suspension (*Hartveit* 1967) and long term intraperitoneal treatment with 1 ml 10 per cent polyethylene glycol 3 times per week does not appear to have adverse general effects on our mice (*Hartveit* 1969)

The present findings suggest rather that it is the host tumour relationship that has been upset by treatment In the control mice growth was infiltrative in type There was no sharp borderline between tumour transplant and the surrounding normal tissues The transition area was filled with a copious acute inflammatory exudate and the blood vessels in the surrounding tissues showed corresponding inflammatory changes

This response is unlikely to be due to a bacterial contaminant in the tumour cell suspension in the control group as the experimental group was injected from the same suspension as the controls on each occasion Capillary permeability increasing factors are known to be present in the ascitic fluid from such tumours (*Thunold* 1965) but in the present case so little tumour ascitic fluid was injected that it is unlikely that the small amounts of these agents present in it could have an effect that was so widespread and so longlasting and in any case the

same amount of fluid was injected in control and treated groups If the fluid is not responsible we are left with the tumour cells themselves (plus any intergral component i e virus they may carry)

In the untreated animals the presence of transplanted tumour cells was followed by the development of an inflammatory response Such cells also elicit an inflammatory response on intraperitoneal transplantation—with the formation of an ascitic tumour in consequence (*Hartveit* 1965 a) The extent of the inflammatory response is related to the number of tumour cells that undergo immunological lysis after transplantation Following tumour cell lysis exudate formation occurs This exudate collects as ascites in which the remaining tumour cells thrive protected from further immunological lysis by the anticomplementary nature of the inflammatory exudate (*Hartveit* 1965 b *Hartveit* 1966)

However if the tumour cells are transplanted subcutaneously the exudate will not have the opportunity to collect to this extent The fluid will tend to diffuse away while the more solid remains will undergo organization This is demonstrated at the edge of the tumours in the present experiment where areas with fresh unorganized exudate were found adjacent to areas where organization was well under way

In contrast to the orderly arrangement of the tumour cells parallel to the surface of the transplant in treated tumours the tumour cells abutting on to this inflammatory exudate surrounding the transplant in the control mice showed no sign of orderly arrangement The tumour cells appeared to be scattered at random throughout the exudate and to be carried with it into the surrounding tissues The nutritional requirements of the tumour cells were obviously amply supplied by this exudate and enough oxygen was apparently present to meet their needs as mitoses were frequent Where organization of the exudate had occurred the tumour cells seemed to have been left high and dry between the newly formed fibroblasts and capillaries Once more their nutritional requirements were obviously satisfied as mitoses were frequent here too

The ability to elicit a stroma and so secure a blood supply thus seems to be dependent on the primary ability to evoke an inflammatory response The present findings suggest that in the absence of an inflammatory response local tumour growth is doomed to be of expansive type In its presence infiltrative growth becomes possible

To achieve an inflammatory response interaction between host and tumour is essential the tumour cells supplying the irritative factor to which the host reacts Failure of either of these processes may be responsible for failure in exudate formation and/or organization

In the polyethylene glycol treated mice the inflammatory response has clearly failed to become established In addition treatment with polyethylene glycol has changed the histological response of both the spleen and lymph nodes to tumour transplantation If as has been

discussed previously the inflammatory stimulus is derived from immunologically injured tumour cells changes in immune response consequent to these histological changes in the reticuloendothelial system need to be taken into consideration

The tumour may fail to elicit an inflammatory response as the host fails to supply the factors *i e* specific antibody and/or complement required for immunological lysis

Polyethylene glycol is known to potentiate immunological lysis *in vitro* It has been suggested that it acts indirectly by forming a loose complex with gamma globulin that in turn traps complement This complement is not fixed in the sense that it is inactivated but may on the contrary be so placed that reaction with antibody is facilitated (see *Hartveit* 1967) If binding of complement occurs intravascularly in polyethylene glycol treated mice it is unlikely that such large complexes *could pass into the extravascular space This could lead to* extravascular decomplementation As *Willoughby et al* (1969) have recently *pointed out complement plays an important part in inflammation* be it of specific or non specific type In the present case all the ingredients required for immunological lysis of the tumour cells are normally available in vivo It is tempting to postulate that lack of available complement due to intravascular trapping may be responsible for the lack of inflammatory response round the tumour transplants in the treated mice

The probability arises from these findings that the ultimate means of achieving infiltrative growth may be given to the tumour cells by the host As such it would be an acquired characteristic not an inborn behaviour disorder peculiar to malignant cells

This hypothesis implies that

1 The cell population involved differs antigenically from its host (The potential malignant cell is thus initiated by factors capable of leading to genetic change *e g* chemicals viruses ionizing radiation)

2 The host is capable of responding to the antigenic stimulus with humoral antibody production and of mounting an inflammatory response to subsequent immunological lysis

3 Cell proliferation within the population is capable of outstripping cell death due to immunological lysis

If these conditions are fulfilled the host may be capable of endowing that cell population with the means of infiltrative growth Working with transplanted Ehrlich carcinoma in our *untreated mice these* conditions are fulfilled The tumour is an allograft to which humoral antibody is formed (*Thunold* 1968) Immunological lysis takes place in the early stages of growth but cell proliferation finally outstrips this lysis (*Hartveit* 1963)

Cellular immunity to tumour growth has received much attention recently (see *Alexander & Fairley* 1967) However experience assures us that immunity to spontaneous tumour growth whether in man or in

animals is a rare exception if it indeed occurs Further *Moller s* (1963) finding that humoral antibody can effectively prevent the recognition of tumour cells by primed host lymphocytes suggests that the cellular response if established may be doomed to failure The latter is supported by the failure of human malignant tumours in general to evoke a local cellular immune response

While cellular immunity has thus failed to establish its place in the physiopathology of tumour growth the probability that humoral immunity may be of aetiological significance has now to be considered

How far conditions similar to those found with the Ehrlich carcinoma prevail in spontaneous tumours remains an open question The finding that increase in tumour size is not directly proportional to the rate of tumour cell proliferation in malignant human neoplasms (see *Refsum* 1968) leads to the assumption that cell death is present here also While it is unusual to find a cellular inflammatory exudate round actively growing tumour tissue in human material the possibility of increased permeability of the surrounding vessels needs further investigation *Burgess & Sylven s* experimental finding (1962) of increased protein content in tumour interstitial fluid coupled with *Cater & Wallington s* (1968) demonstration of the increased sensitivity of newly formed tumour vessels to chemical mediators of inflammation provide additional reasons to investigate this further

SUMMARY

Inhibition of growth of Ehrlich s carcinoma by oral treatment with polyethylene glycol 4000 is accompanied by a marked reduction in the inflammatory response around subcutaneous tumour transplants In the absence of formation of an inflammatory exudate tumour growth was expansive and not infiltrative in type

The lymph nodes in treated animals showed marked proliferation while the spleen was atrophic Such changes were not present in the untreated controls It is suggested that treatment with polyethylene glycol may have upset the immunological balance in host tumour relationship and that subsequent changes in response may be ultimately responsible for the difference in the subcutaneous growth of these tumour transplants

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THE INHIBITORY EFFECT OF ORAL POLYETHYLENE GLYCOL 4000 ON THE PHAGOCYTIC ACTIVITY OF THE RETICULOENDOTHELIAL SYSTEM, RELATED TO TUMOUR TRANSPLANTATION IN MICE

By

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Received 21 vi 69

Polyethylene glycol is a long chain polymer which has the ability of potentiating the action of complement in vitro in both the immune haemolytic (*Mcl ickar* 1949) and immune oncolytic (*Hartveit* 1967a) systems When given orally or intraperitoneally it has been shown to inhibit the subcutaneous growth of Ehrlich s carcinoma in mice (*Hartveit* 1969 1970) Oral treatment with polyethylene glycol 4000 is accompanied by proliferative changes in the lymph nodes with active germinal centres and paracortical proliferation while the spleen remains small and inactive compared to the spleen in tumour bearing mice that have not been given polyethylene glycol

The mode of action of polyethylene glycol on tumour growth in vivo is as yet unexplained though it may be related to its ability to alter the reactivity of complement (see *Hartveit* 1970) At the same time polyethylene glycol s physical character and high molecular weight make it likely that it will be taken up by the reticuloendothelial system The following experiments were set up to see if this were so using estimation of carbon clearance as a measure of the phagocytic ability of the reticuloendothelial system as a whole

MATERIAL AND METHODS

Mice aged 5 months of the closed colony kept at this Institute were used

Polyethyl ne gly ol 4000 was given as a 20 per cent solution in the drinking water ad lib

The tumour used was the Ehrlich ascites carcinoma kept by serial intraperitoneal transplantation in our mice Whole tumour ascites from a 10 day transplant was injected subcutaneously on th back at a dosage of 0 05 ml per mouse

The phagocyt c activity of the reticuloendothelial system was determined by

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carbon clearance assessed by direct microscopical evaluation of blood samples (*Hartveit et al* 1967b) A carbon dosage equivalent to 8 mg/100 g body weight was used

Experimental Procedure

The carbon clearance time was determined in male and female mice

1) in the absence of treatment
2) following treatment with oral polyethylene glycol
3) following tumour transplantation alone.
4) following tumour transplantation and treatment with oral polyethylene glycol starting from the time of transplantation

At stated intervals see Figs 1 and 3 the clearance time was recorded and the mice were killed The spleens were removed fixed in formalin and later weighed after standing for one hour in the air

RESULTS

Treatment with Polyethylene Glycol per os

The clearance time increased in both sexes when the mice were given polyethylene glycol to drink (Fig 1) The positive correlation between clearance time and days of treatment was statistically significant in both sexes 0 001 > P r ♂ = 0 7977 r ♀ = 0 7538

The spleen weight on the contrary decreased in both sexes on treatment with polyethylene glycol (Fig 2) The negative correlation between spleen weight and days of treatment was statistically significant in both sexes 0 001 > P r ♂ = — 0 7246 r ♀ = — 0 7625

There is thus in both sexes a negative correlation between clearance

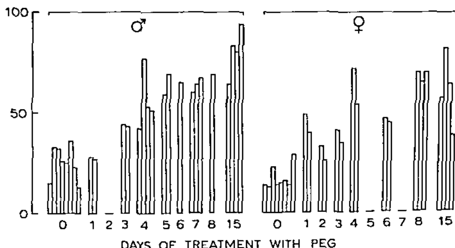


Fig 1

The individual clearance times in untreated mice (0 days) and mice treated with polyethylene glycol (PEG) related to days of treatment

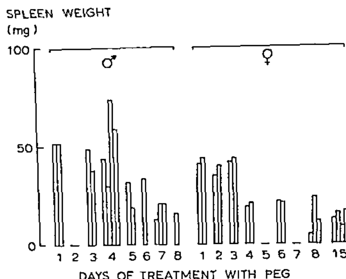


Fig 2
The individual spleen weights in polyethylene glycol (PEG) treated mice related to days of treatment

time and spleen weight r ♂ = —0 6472 (*0 01 > P > 0 001*) r ♀ = —0 7625 (*0 001 > P*)

Tumour Transplantation

The *clearance time* following subcutaneous tumour transplantation was similar to that in untreated mice (see day 0 Fig 1) at 3 days but then dropped (Fig 3) The overall difference i e reduction in clearance time from that in the untreated mice was statistically significant ♂ *0 01 > P > 0 001* ♀ *0 02> P > 0 01*

The spleen weight showed a marked rise by the 8th day followed by a drop both rise and fall being more pronounced in the males than in the females (Fig 4)

Treatment with Polyethylene Glycol and Tumour Tranplantation

The clearance time fell slightly in both sexes with time (Fig 3) but all in all did not differ significantly from that in untreated mice (see day 0 Fig 1)

The spleen weight showed the same pattern i e a rise by day 8 followed by a fall in both sexes as was seen in the mice given tumour alone (Fig 4) However although the pattern was similar the spleens were much smaller The overall means ± S D in the mice given tumour alone were ♂ 131 ± 128 mg ♀ 112 ± 59 mg while the corresponding figures for those given tumour plus polyethylene glycol were ♂ 43 ± 18 mg and ♀ 53 ± 22 mg

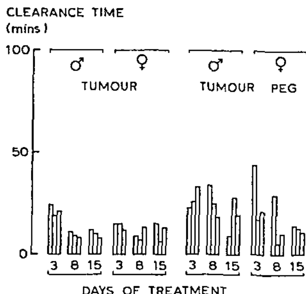


Fig 3

The individual clearance times in mi e treated with subcutaneous tumour and in mice treated with tumour and polyethylene glycol (PEG) related to days of treatment

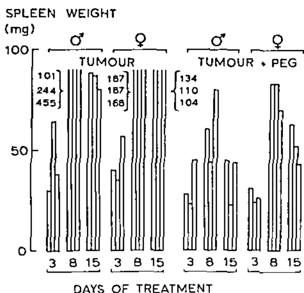


Fig 4

The individual spleen weights in mice treated with sub utaneous tumour and in mice treated with tumour and polyethylene glycol (PEG) related to days of treatment

DISCUSSION

The present experiments demonstrate that mice given polyethylene glycol 4000 per os become progressively less able to remove carbon particles from their blood stream That is to say the phagocytic activity of the reticuloendothelial system is depressed

This reduction in phagocytic activity is accompanied by a reduction in the size of the spleen As the spleen contains a high proportion of the cells responsible for the phagocytosis of carbon particles in the body this reduction in splenic weight is probably to a great extent responsible for the delayed clearance time recorded

A similar reduction in spleen weight and in carbon clearance has also been found in mice given polyethylene glycol 4000 intraperitoneally (personal observation) It is thus likely that the substance is absorbed when given per os and the possibility of an indirect effect on the reticuloendothelial system can probably be excluded Blockade of the phagocytes by polyethylene glycol can well be expected due to the physical characteristics of the molecule However although the experiment demonstrates that phagocytic activity is decreased it cannot tell us whether the reduction is in fact due to prior blockade of the phagocytes to a reduction in their number or to a mixture of these two processes

In untreated tumour bearing mice the phagocytic activity of the reticuloendothelial system was increased This increase was accompanied by a gross increase in spleen weight as has been reported previously (*Hartveit* 1966) The increase was maximal at 8 days after transplantation and then dropped This finding is also in keeping with previous studies from this Institute (*Thunold* 1968) For the reasons mentioned above it is reasonable in this case to attribute the reduction in carbon clearance to increased phagocytic capacity of the spleen

The tumour bearing mice treated with polyethylene glycol showed clearance times and spleen weights within the range of normal (Figs 3 and 4) *i e* between these two extremes This suggests that two opposing forces have been at work and that the stimulating effect of transplantation cancelled out the depressive action of polyethylene glycol

While these clearance times are not surprising viewed in the light of the spleen weights recorded they call for comment when the previous finding that tumour growth is reduced in polyethylene glycol treated mice is also taken into consideration

The reactivity of the lymphoid and phagocytic elements of the reticuloendothelial system are usually assumed to go hand in hand However in the present experimental situation there is reason to believe that this may not be so as concommitant lymph node hyperplasia and splenic atrophy have been reported in tumour bearing mice treated with oral polyethylene glycol (vide supra)

This odd combination of findings was accompanied by a reduction

in tumour growth compared to that in untreated tumour bearing mice in which proliferation of the lymphoid elements was recorded in both the lymph nodes and spleen The present work shows that in the case of untreated tumour bearing mice such increased activity was accom panied by increased activity of the phagocytic elements Stimulation of the lymphoid elements in the lymph nodes and spleen in the presence of stimulated phagocytosis thus appears to be accompanied by better tumour growth than stimulation of the lymphoid elements in the lymph nodes and depression of the phagocytic elements of the reticulo endothelial system However this is obviously not the whole story as qualitative differences in the histology of the lymphoid tissue reaction were also recorded and need further study

The role of the reticuloendothelial system in relation to tumour growth is variously described in the literature For example *blockade* by trypan blue will permit the growth of tumours in genetically in compatible hosts (*Ludford* 1931 *Andervont* 1936) *Depression* by cortisone may have a similar effect and has been extensively used in studies on xenotransplantation (see *Toolan* 1953) *Depression* or '*exhaustion* in the course of tumour growth has been evoked to ex plain the survival of antigenic tumours in their host of origin (see *Alexander & Fairley* 1967) and *inactivation* by irradiation is also a common experimental tool *Stimulation* by BCG vaccine on the other hand is said to have an inhibitory effect on tumour growth (*Old et al* 1959)

While the findings quoted may be clear cut they are difficult to interpret as the reticuloendothelial system as a whole is considered and not the interplay of its various elements With the recent recogni tion of the significance of paracortical versus follicular proliferation of the lymphoid elements (*Turk & Oort* 1967) the role of thymic dependence (*Parrott et al* 1966) and the part played by macrophages in the initiation of immune responses (*Dumonde* 1967) much of the pre vious work on this system is in urgent need of revision particularly as far as its relationship to tumour growth is concerned

SUMMARY

Oral treatment with polyethylene glycol 4000 was found to decrease the phagocytic activity of the reticuloendothelial system in mice as measured by carbon clearance This is probably related to the decrease in spleen weight also seen in these mice In tumour bearing mice (sub cutaneous Ehrlich carcinoma) given polyethylene glycol the phagocytic activity and spleen weight was similar to that in normal mice However as untreated tumour bearing mice showed a decrease in carbon clear ance and an increase in spleen weight these apparently normal values are probably the result of a combination of stimulation and depres sion of the reticuloendothelial system

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A SIMPLE MICRO CINEMATOGRAPHIC SYSTEM

By

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Received 28 vi 69

During micro cinematographic observation of mammalian cells it is usually significant to keep temperature constant since variation will influence synthetic activity in the cells examined A variety of micro cinematographic systems have been constructed for this purpose (1) of which most are rather complicated and expensive The systems on record (1) appear all to have been based on incubators surrounding the microscope more or less completely Most such arrangements are space consuming and difficult to operate making the microscope useless for other purposes Temperature recordings tend to be incorrect if made in the incubator and not in the cell chamber itself and consequently it is not feasible to measure changes in the chamber temperature *e g* during exposure to transmitted light As this communication reports this may cause serious disadvantages

ELEMENTS

Microscope Carl Zeiss photomicroscope I (light source 6 V 60 W Wolfram) equipped with phase contrast condensor Z VII and standard phasecontrast objectives

Culture chamber The chamber resembles in various ways the Prior tissue culture chamber as obtained from Fa Bie & Berntsen Copenhagen

It consists of two standard microscope cover glasses separated by a O section silicone rubber ring These were originally contained in an aluminum housing but in our modification replaced by a stainless steel housing which by pressure on the silicone ring *renders the chamber air tight and is resistent to the cleaning medium* RBS 25 Introduction of cell cultures and/or medium is made through injection needles piercing the housing and the silicone rubber ring In the same way it is possible to maintain a constant gas flow through the chamber The chamber is placed on top of a Carl Zeiss heating stage for temperatures ranging from 35 –43 C (86 –109 4 F) Originally the stage was equipped with a reading thermometer and temperature was regulated manually Since temperature variation was found to exceed the permissible the culture chamber was equipped with a built in contact thermometer (Jacob Glasteknik Copenhagen) for automatic temperature regulation Controlled in this way the temperature of the steel housing was set on 38 5 C.

Electronic timer For the exposure of the culture chamber to transmitted light with preselected intervals an electronic timer was constructed The diagram of the timer is shown in Fig 3

An astable multivibrator gives of voltage peaks in series which slowly discharges

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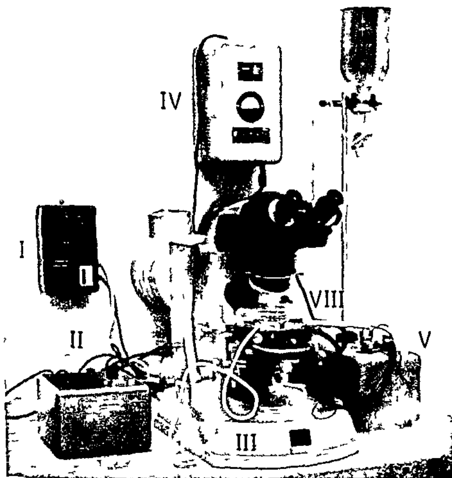


Fig 1 a
A photographic presentation of the microscope m ιntc l as micro-cinematograph

the condensor on a large RC circuit (50 Mω 64 μF Tantal) The cathode of a diode i connected to the RC circuit and the anode to a variable voltage divider As soon as the cathode potential exceeds the negative preselected level at the anode the diode becomes conducting trigging the m nostaḃle multivibrator via a con densor So the multiv l ator is kipped relea ing a relav connected as collector load in one of the tran istors A variable RC rcuit connected to the l ase of this transistor keeps the m nostable multivil rator in this p o ition for a preselected interval An off conta t at th relay triggers the Carl Zeiss photo timer Another contact charges the RC ircuit which once m re is slowly lischarged By this arrangement frames can l e tak n once every 1 10 20 60 nd 120 minutes

METHODS

A schematic presentati n f the test arrangement used for temperature recordings fr m the interior of th ell hamber is sh wn in Fig 4

An iron-constantan tl r feeler vas intr duced into the cl aml er through a

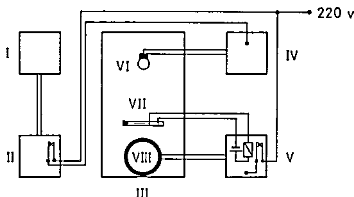


Fig 1 b

The electric wiring of the system I Electronic timer II Carl Zeiss phototimer III Carl Zeiss photomicroscope I IV Power supply for the wolframlamp (6 V 60 W) V Power supply for the heating stage VI Wolframlamp VII Contact thermometer VIII Heating stage The relay contact on the exposure relay cuts out the power supply for the wolframlamp The relay circuit controlling the power supply of the heating stage is cut out by the contact thermometer The electric timer switch on the exposure contact of the phototimer

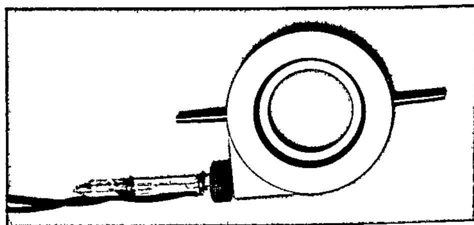


Fig 2

Photographic presentation of the culture chamber The chamber is made of stainless steel The top can be screwed off A 32 mm diameter circular glass cover slip is placed in the base of the chamber A silicone O ring is then placed on it and needles (1 2 × 40 mm) to carry the gas flow and/or act as an air leak when the chamber is filled or emptied is inserted through the two small holes in the base of the housing and through the O ring which is supported meanwhile with the tip of a pair of forceps Another cover slip is then placed on top of the O ring followed by the chamber top which is lightly screwed down to contact with the cover slip

13 gauge needle In order to keep the assay as close to normal conditions as possible the chamber was filled with Eagle MEM medium As reference temperature an ice bath equilibrated for at least 2 hours in a thermo bottle was used The potential provided by the thermo feeler was amplified 1000 times by Gleichstroms Messver starker KNICK model MV and led to a Honeywell model υ12M recorder Two serially connected quicksilver cells (1 35 V) were stored in an icebath and inserted between

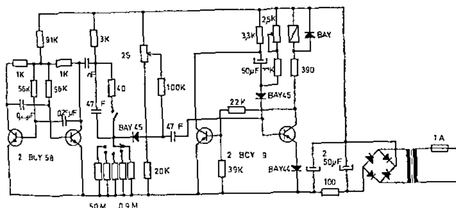


Fig 3

Diagram of the electronic timer For details see elements

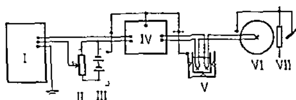


Fig 4

The test arrangement for temperature recordings from the interior of the culture chamber I Recorder II Variable potentiometer III Quick silver cells IV Amplifier V Ice bath VI Iron constantan thermofeeler VII Heating stage

the amplifier and the recorder through a 100 kohm variable potentiometric voltage divider This was done in order to depress the signal All components were screened and connected to electrical earth except the recorder mass which was connected to ground

RESULTS

First the average temperatures along the diameter of the culture chamber were recorded For this purpose the diameter was divided in 1 mm sections Measurements were performed corresponding to each of these sections In consequence of the heating of the chamber through the surrounding circular housing the temperature measured at any point will represent that of all points at the same distance from the perifery Secondly the temperature variations in each individual point were recorded The results are shown in Fig 5

It is seen that the temperature gradually increases from the centre toward the perifery of the chamber The total variation is equal to 1 08 C However it should be mentioned that the central 6 mm are kept constant on 37 C The figure further shows that the temperature variation at the perifery is ± 0 05 C (corresponding to the contact

will effectively reduce the light induced temperature variations so that the full value of a strict temperature controlled micro cinematographic system is attained The proposed system furthermore has the advantages of beeing space saving easily operated and inexpensive In comparison with the price of about $ 11 000 for a complete micro cinematograph the cost of the present system besides ca $ 4 500 for a photo microscope will not exceed $ 500 Another advantage is that its flexibility (dismounting is done by loosening a single screw) permits the use of the microscope for other purposes

SUMMARY

The construction of a simple spacesaving and inexpensive cinematographic system is reported Transmitted light causes significant temperature variations in the culture chamber The importance of such variations is briefly emphasized The temperature variations can be decreased by the insertion of a heat absorber between the light source and the condensor

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SPONTANEOUS AND EXPERIMENTAL THROMBOSIS IN THE MOUSE PLACENTA

By

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Received 13 vi 69

In a previous study it was shown that the deposits on the syncytium of the normal human placenta are maternal fibrin thrombi formed from platelet thrombi (17) Whether the platelet aggregates are formed on the wall of the maternal blood space or whether they are primarily formed in the flowing blood and thereafter attach to the vascular lining could not be decided The particular pattern of blood flow in the intervillous space was assumed to be responsible for placental thrombosis the high values of coagulation factors generally and locally and the reduction of fibrinolytic activity during pregnancy were pointed to as contributing factors (17) The relative importance of these three sets of thrombogenic factors could not be clearly defined

In the present study the placentas in 16 days pregnant mice were examined Though the placenta in mouse differs from that in human subjects there are certain similarities with regard to structure and circulatory flow pattern One should therefore expect to find thrombi also in the normal mouse placenta In an attempt to enhance the formation of thrombi animals were made hypercoagulable by ellagic acid which has been found to activate factor XII (Hageman factor) (20)

The purpose of the present study is to explore the following problems

1 Is thrombosis within the maternal blood channels a normal feature even of the mouse placenta? If so where are the thrombi located? How are they formed and what is their fate?

2 If there is a tendency to thrombosis in the mouse placenta is it caused by a generalized thrombogenic propensity during pregnancy or does it depend on local factors?

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The determination of fibrinogen by dr *H C Godal* Ullevål Hospital Haematological Research Laboratory Dept IX University of Oslo is gratefully acknowledged

MATERIALS

Animals Altogether 205 virgin female albino mice were used They were 55–77 days old and weighed 25–33 g (mean 28 1 g) The animals were kept under artificial light in plastic cages the floor of which was covered by saw dust They were fed a standard diet (SIFI Norwegian standard stock diet No 1 mice and rats) and tap water *ad libitum* Animals taken out for mating were kept single and the males were put in with the females in the morning After 24 hours the males were removed That day was designated day one of pregnancy if the animal one week later showed increasing weight All experiments were performed at day 16 of pregnancy The body weight was then 39–55 g (mean 46 0 g)

Ellagic acid Supplied by K and K Lab Inc New York U S A The ellagic acid was dissolved in buffered saline to a concentration of 4×10^{-4} M as described by *Nordoy & Chandler* (18)

Buffered saline A 0 9 per cent solution of NaCl in distilled water was buffered with 10 per cent tris (hydroxymethyl) aminomethane HCl 0 15 M to a final pH 7 2 and used as diluent

Heparin Heparin 5 per cent AL Oslo Norway containing 5000 I U per ml was used in all experiments it was diluted in buffered saline to a final concentration of 50 I U per ml

METHODS

Anaesthesia The animals were anaesthetized by intraperitoneal injection of *Nembutal Veterinary* (Abbott Lab Ltd Queenborough Kent England) 5 mg per 100 g body weight

Experimental procedure The number of pregnant animals and the duration of the experimental period in the various groups of animals are summarized in Table 1

TABLE 1
Experimental Groups

Injection given	No of animals	No of placentas	Time of sacrifice after last injection
Pregnant			
Untreated	10	82	–
Ellagic acid	10	78	3 min
Ellagic acid	10	100	10 min
Ellagic acid	10	97	24 hrs
Heparin and ellagic acid	5	45	10 min
Heparin and ellagic acid	5	38	24 hrs
Buffered saline	5	45	3 min
Buffered saline	5	40	10 min
Buffered saline	5	50	24 hrs
Non pregnant			
Untreated	5		–
Ellagic acid	5		3 min
Ellagic acid	5		10 min
Ellagic acid	10		24 hrs
Buffered saline	5		3 min
Buffered saline	5		10 min
Buffered saline	5		24 hrs
	105	575	

Grained barley 35 per cent grained oat 15 per cent grained wheat 12 per cent soya meal 12 per cent herring meal 10 per cent grass meal 5 per cent dried skimmed milk 10 per cent salt and mineral and vitamin mixtures 1 per cent

Ellagic acid was given into the tail vein in a dosage of 1 ml (4×10^{-4} M) per 100 g over a period of 30 seconds

In some experiments heparin in a dosage of 10 I U per 100 g body weight was given into the tail vein in a course of 10 seconds Immediately after the administration of heparin ellagic acid was injected through the same needle over a period of 30 seconds

In control animals a comparable volume of buffered saline was substituted for the solution of ellagic acid

No unintended deaths occurred among the animals

The organs for microscopical examination were removed in the anaesthetized animal for immediate fixation In the group examined 24 hours after ellagic acid the animals were re anaesthetized and the organs removed for fixation

Fixation and staining methods for light microscopy All placentas kidneys liver lungs and heart from the animals were fixed over night in formaldehyde mercuric chloride acetic acid (8 per cent formaldehyde with 5 per cent mercuric chloride To this solution was added 5 per cent glacial acetic acid before use) The specimens were embedded in paraffin and cut at 5 μ The placentas were cut transversely through the middle part One section from each organ was stained routinely with Masson s haematoxylin erythrosin saffron method (22) Selected sections were stained with Lendrum s Martius scarlet blue method (MSB) (15)

Examination by light microscopy All sections were examined without knowledge of the treatment given to the animal in question

Fixation and staining method for electron microscopy Two animals sacrificed 10 minutes after treatment with ellagic acid and buffered saline respectively were used Tissue blocks from one placenta in each animal were fixed for 1½–2 hours in chilled isotonic 1½ per cent glutaraldehyde in M/20 phosphate buffer (pH 7 4) and post fixed for 1¼ hour in 1 per cent isotonic osmic tetroxide (3) and embedded in Epon 812 One micron thick sections were cut on a Huxley ultramicrotome (Cambridge Inst Co) and stained with toluidine blue for orientation by light microscopy Suitable areas were selected and ultrathin sections stained with uranyl acetate and lead citrate (21) They were examined in a Zeiss EM 9 electron microscope

Bleeding time 3 minutes after the injection of ellagic acid or buffered saline the tail of the anaesthetized mouse was cut with a sharp razor blade approximately mm from the tip after it had been prewarmed in 0 9 per cent NaCl at 37 C for one minute. The animal was placed on a horizontal surface with the tail hanging down into a bath containing 0 9 per cent NaCl at 37 C and the time was measured from the moment the tail was cut until the bleeding into the saline had stopped for one minute (18)

Whole blood clotting time Immediately after the bleeding time had been measured the tail was cut near the root and a capillary pipette was filled with blood At short intervals a small piece of the pipette was broken off The clotting time was recorded as the time from the filling of the pipette until the first appearance of a fibrin thread

Fibrinogen Anaesthetized animals were decapitated and 0 9 ml of blood were allowed to flow into a plastic tube containing 0 1 ml of citrate anticoagulant Fibrinogen was measured by the method of *Schneider* (24) modified by *Hjort* (7)

Statistical methods Statistical evaluation of group differences was made by the Wilcoxon rank test (26)

THE LABYRINTHINE PLACENTA IN THE MOUSE (1)

The labyrinth is the major location of exchange of metabolites between mother and foetus In this part of the placenta the foetal and maternal vessels intermingle The foetal vessels are of capillary size and lined by endothelium They are surrounded by small ramifying maternal blood channels the trophoblastic tubules (Fig 1)

The layer of the placenta which is closest to the decidua basalis is called the giant cell layer and corresponds to the floor of the human placenta It is a plate of cells arranged in irregular sheets and cords The cells are of two types A large type the giant cells the cytoplasm of which is dense and runs out in thin long processes There i[illegible] ly one large nucleus The other type of cells is smaller though the size and [illegible] aries somewhat Their cytoplasm is basophilic and con-

tains abundant glycogen The nuclei are small and dark These cells are usually arranged in clusters

The decidua of the mouse uterus is relatively scant compared with that of the human uterus

The uterine and decidual arteries unite into a single central artery for each placenta The artery passes through the placenta and ends in the placental roof" in a system of lacunae lined with syncytium (lacunae of the roof") From these the blood passes through the narrow trophoblastic tubules which are lined by a layer of syncytiotrophoblast (the tubular area) The trophoblastic tubules empty into wide lacunar spaces of the giant cell layer mostly lined with endothelium like cells (lacunae of the giant cell layer) The maternal blood is drained through endothelium lined maternal veins which leave the placenta peripherally

RESULTS

The following intravascular bodies of thrombotic nature were distinguished

1 *Loosely Packed Platelet Aggregates*

Loosely aggregated clumps of granular platelets which appeared to be either floating free in blood or occasionally loosely attached to the wall of the maternal blood channels Such aggregates frequently occurred in the uterine veins (Fig 2) in the lacunae of the roof and in the lacunae of the giant cell layer

Loosely arranged platelet aggregates were also regularly observed in the small pulmonary vessels and occasionally in the right ventricle and the coronary arteries of the heart and in the liver veins A few were seen in the kidney vessels

Since the significance of these aggregates is uncertain they have not been included in the following analysis

2 *Dense Platelet Aggregates*

Densely packed platelet aggregates formed more well defined bodies composed of platelets in intimate contact with each other (Figs 3 and

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Figs 1–4

Fig 1 Transverse section through the middle part of a labyrinthine placenta in a 16 days pregnant mouse LR lacunae of the roof T tubular area G giant cell layer LG lacunae of the giant cell layer which often extend into the tubular area D decidua M myometrium U umbilical vessels Arrow placental artery HPS × 30

Fig 2 Loosely packed platelet aggregates in a decidual vein Some of the aggregates are in contact with the endothelium MSB × 500

Fig 3 A dense platelet aggregate is occluding a trophoblastic tubulus in the tubular area Syncytial nuclei are pycnotic MSB × 500

Fig 4 Two neighbouring lacunae of the giant cell layer An apparently freely floating platelet aggregate is seen in the smaller lacuna in the lower part of the picture Many of the platelets are swollen and the darker outline indicates traces of fibrin (arrow) The larger lacuna in the middle of the picture contains a thrombus mostly composed of fibrin and apparently attached to the vascular lining Red stain of fibrin appears dark grey MSB × 500


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4) Single platelets were often difficult to distinguish In some aggregates the platelets were granular In others many of the platelets appeared to be swollen and had an empty interior (Fig 4) Traces of fibrin at the periphery could be distinguished in some of the aggregates

3 *Platelet Fibrin Masses*

Platelet aggregates with distinct fibrin membranes at or near the periphery of the individual platelet aggregates (Figs 12 and 13)

The dense platelet aggregates and the platelet fibrin masses either appeared to be floating free in the blood stream or they seemed to be attached to the vessel wall

4 *Fibrin Thrombi* (Figs 4 7 8 9 10 and 11)

Thrombi predominantly composed of densely packed fibrin fibrils often with what appeared to be remnants of platelet masses in between the fibrin strands Occasionally eosinophilic masses were observed in which no fibrillar structure could be distinguished except near the surface The interior of these masses was either hyaline or granular

The fibrin thrombi were always attached to the vessel wall as mural thrombi

5 *Hyaline Microthrombi* (Fig 6)

In the small trophoblastic tubules structureless bodies with an appearance similar to that of hyaline microthrombi (25) were seen in a few cases

Thrombosis in the Placenta and in the Myometrial Vessels of Untreated Mice

Dense platelet aggregates platelet fibrin masses and fibrin thrombi were observed in all parts of the maternal vessels of the placenta except the placental artery they were never observed in foetal vessels In the myometrium they were seen in the veins and not in the arteries

Dense platelet aggregates were seen in small trophoblastic tubules of all placentas They were preferentially located to the upper part of the tubular area Most of the dense platelet aggregates occluded the lumen (Fig 3) The cytoplasm of the syncytium bordering the occluded tubules appeared occasionally to be shrunken and the nuclei showed pyknosis or karyorrhexis (Fig 3)

Dense platelet aggregates were occasionally seen in the lacunae of the roof and in the lacunae of the giant cell layer (Fig 4) More rarely they occurred in the uterine veins (Fig 5) In most instances these dense platelet aggregates were floating free although sometimes they appeared to be in contact with the vascular lining without occluding

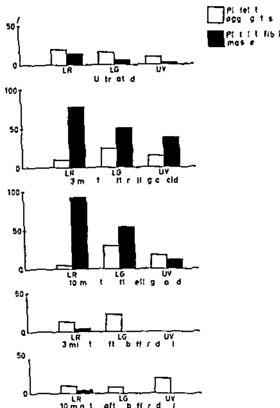


Fig 5

The frequency of apparently freely floating dense platelet aggregates and platelet fibrin masses found in the lacunae of the roof (LR) lacunae of the giant cell layer (LG) and uterine veins (UV) (mean percentage of placentas)

the lumen The lining cells at the sites of contact most frequently seemed unaltered

Platelet fibrin masses were seen in the lacunae of the roof in some cases More rarely they were found in the lacunae of the giant cell layer or in the myometrial veins (Fig 5)

Fibrin thrombi were regularly observed in the lacunae of the roof (Figs 7 and 8) Sometimes they extended a short distance into the proximal end of the tubuli but not in the remaining part of the tubular area Fibrin thrombi were frequently seen in the lacunae of the giant cell layer (Fig 9) and in the uterine veins (Figs 10 and 11) as well

In the lacunae of the roof the fibrin thrombi were regularly located to curved parts or small pockets (Fig 7) The underlying syncytium was occasionally altered as described above (Fig 8)

Underneath fibrin thrombi in the lacunae of the giant cell layer the endothelium like cells were sometimes lacking and fibrin thrombi were in direct contact with trophoblastic cells Occasionally the fibrin

appeared to merge with similar masses between the cells (Fig 9) In the myometrial veins the endothelium underneath the thrombi was often missing (Figs 10 and 11) and the fibrin of the thrombi was continuous with fibrin like masses between the smooth muscle cells (Fig 11)

Alterated lining cells not associated with thrombotic materials were rarely seen

In the placenta there was no invasion of leucocytes into the thrombi and signs of organization were not observed Clear morphological evidence of fibrinolysis was lacking Occasionally the surface of the thrombi was partly or totally covered by syncytium (Fig 8)

In contrast to the situation in the placenta leucocytes were often observed within the thrombi in the uterine veins (Fig 10) Regularly endothelium covered parts of these thrombi (Fig 11)

Thrombosis in the Placenta and the Myometrial Vessels after Injection of Ellagic Acid

Sacrifice at 3 and 10 Minutes after Injection of Ellagic Acid

Fig 5 shows the frequency at which platelet fibrin masses were seen in the different areas of the maternal blood channels (dark columns) The percentage of placentas within each mother animal showing platelet fibrin masses in each location was determined From these values mean percentages were calculated for each location within each experimental group

In animals killed 3 and 10 minutes after injection of ellagic acid there was a marked increase in the number of platelet fibrin masses compared with untreated animals and those treated with buffered

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Figs 6–11

Fig 6 Hyaline microthrombus (arrow) in trophoblastic tubulus in upper part of labyrinth stains as fibrin (red dark grey in picture) MSB × 500

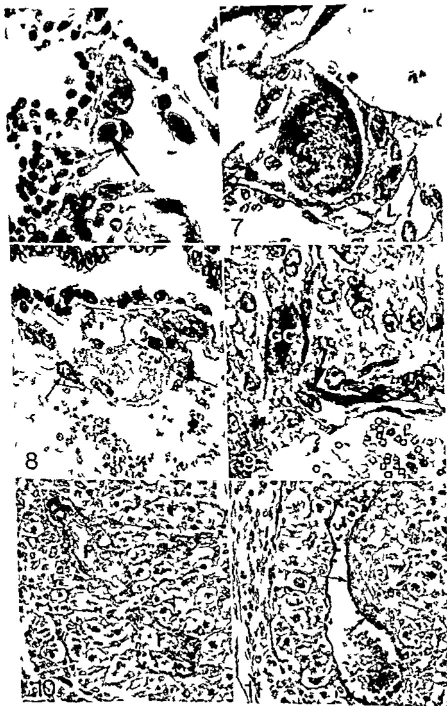
Fig 7 Fibrin thrombus in a pocket of the lacunae of the roof Labyrinth (L) to lower right HPS × 500

Fig 8 Fibrin thrombus in a lacuna of the roof The syncytium (arrows) is in part covering the thrombus The nuclei of the syncytium at the base of the thrombus are partly fragmenting HPS × 500

Fig 9 Thrombus mostly composed of fibrin in a lacuna of the giant cell layer The endothelium like cells are missing and masses with the appearance of fibrin (arrows) are seen between the trophoblastic cells GC giant cell HES × 500

Fig 10 Uterine veins with fibrin thrombi (F) Note several leucocytes within thrombi The endothelium underneath the thrombus is partly lost HPS × 200

Fig 11 Fibrin thrombus in uterine vein The endothelium underneath the thrombus is lost Instead endothelial cells are partly covering the thrombus (arrows) HPS × 200


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saline The differences between the ellagic acid groups and the untreated group and between the ellagic acid groups and the buffer groups are significant for each location The platelet fibrin masses were located exclusively to the lacunae (Figs 12 13 and 14) and the uterine veins Both in untreated and ellagic acid treated animals platelet fibrin masses were most frequently seen in the lacunae of the roof less frequently in the lacunae of the giant cell layer and still less frequently in the uterine veins However the significance of this apparent down stream decrease in frequency is difficult to estimate since in most cases the lacunae of the roof covered a greater section area than the lacunae of the giant cell layer and the uterine veins

In both lacunar areas platelet fibrin masses were somewhat more frequent in the animals killed 10 minutes after ellagic acid injection than in those killed after 3 minutes whereas the reverse relation was seen in the uterine veins None of these differences are statistic ally significant

Most platelet fibrin masses appeared to have been floating free and were surrounded by erythrocytes (Fig 12) a few seemed to be in contact with the syncytium endothelium or mural fibrin thrombi The size of the platelet fibrin masses and the amount of fibrin asso ciated with the platelets had increased from 3 to 10 minutes after ellagic acid injection in all areas (Fig 13) Ten minutes after the ellagic acid injection the lumen of the lacunae of the roof and of the giant cell layer were nearly occluded by platelet fibrin masses (Fig 14) in some cases

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Figs 12–17

Fig 12 Three minutes after injection of ellagic acid A mass composed of platelets and fibrin appears to be floating freely in a lacuna of the roof HPS × 500

Fig 13 Ten minutes after injection of ellagic acid Large platelet fibrin mass in lacuna of the roof HPS × 500

Fig 14 Ten minutes after injection of ellagic acid A lacuna of giant cell layer is nearly occluded by a platelet fibrin mass Arrows red blood cells HPS × 200

Fig 15 24 hours after injection of ellagic acid The labyrinth is necrotic and the lacunae and tubuli are dilated and packed with red blood cells Interstitial bleeding has taken place HPS × 200

Fig 16 24 hours after injection of ellagic acid Necrosis of the placenta The la cunae of the giant cell layer are dilated and filled with platelet fibrin mas ses There is leakage of blood cells and fibrin into the junctional zone (arrow) HPS × 75

Fig 17 24 hours after injection of ellagic acid Thrombus mostly composed of platelets and fibrin in uterine vein Note the absence of endothelial cells underneath the thrombus and the presence of leucocytes within the throm bus HPS × 200

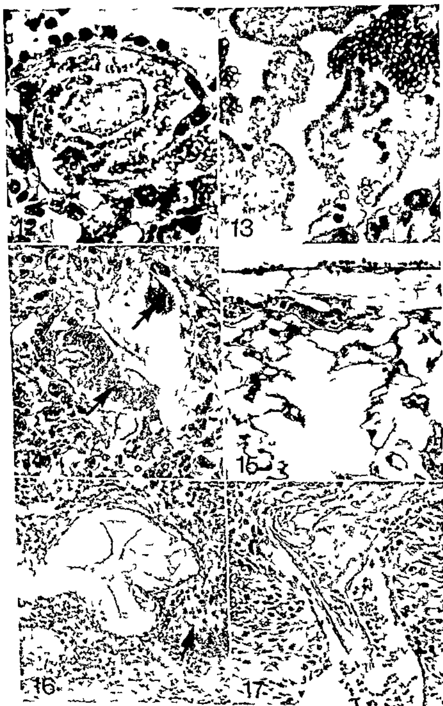

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TABLE 2
Number of Mural Thrombi Predominantly Composed of Fibrin in the Lacunae of the Roof

Injection given	No of animals	Time of sacrifice after injection	Mean number of thrombi	S E §
Untreated	10	–	6 3	± 0 73
Ellagic acid	10	3 min	5 [illegible]	± 0 55
Ellagic acid	10	10 min	5 1	± 0 46
Buffered saline	5	3 min	4 8	± 0 94
Buffered saline	5	10 min	6 3	± 1 75
Buffered saline	5	24 hrs	5 7	± 0 60

Mean of means per mother animal
§ Standard error of the mean

Fig 5 also shows the relative numbers of placentas with only dense platelet aggregates without fibrin in the lacunae and in the uterine veins Dense platelet aggregates that were found together with platelet fibrin masses are not expressed in the figure When the recorded values for only dense platelet aggregates are considered separately no systematic trend of variation among the experimental groups is seen

Neither did the frequency of placentas with mural fibrin thrombi show significant differences among the groups of animals studied (Table 2)

Table 3 shows that in the tubular area there was an unexplained high number of dense platelet aggregates in untreated animals There was no significant difference in the number of dense platelet aggregates after injection of buffered saline or ellagic acid No platelet fibrin masses or mural fibrin thrombi were seen in this area

Thus the main difference between the controls and the ellagic acid treated animals killed after 3 and 10 minutes is in the frequency of platelet fibrin masses in the lacunae and the uterine veins

TABLE 3
Number of Dense Platelet Aggregates Observed in Tubular Area

Injection given	No of animals	Time of sacrifice after injection	Mean number of platelet aggr	S E §
Untreated	10	–	8 0	± 1 45
Ellagic acid	10	3 min	4 2	± 1 55
Ellagic acid	10	10 min	3 6	± 1 10
Buffered saline	5	3 min	6 9	± 2 38
Buffered saline	5	10 min	5 6	± 1 61
Buffered saline	5	24 hrs	4	± 0 22

Mean of means per mother animal
§ Standard error of the mean

Sacrifice at 24 Hours after Injection of Ellagic Acid

In 74 out of 97 placentas the entire placenta was necrotic and 3 placentas showed partial necrosis

In the necrotic placentas (Figs 15 and 16) the number of dense platelet aggregates platelet fibrin masses and fibrin thrombi could not exactly be counted Therefore the group of animals killed 24 hours after ellagic acid injection are not included in Fig 5 and in Tables 2 and 3

The lacunae of the giant cell layer especially those centrally located were considerably dilated and filled with platelet fibrin masses erythrocytes and a few leucocytes (Fig 16) Blood cells had leaked into the surrounding areas of necrotic trophoblast and into the junctional zone between the giant cell layer and the decidua (Fig 16)

In some cases a partial separation of the placenta from the uterine wall was observed

The uterine veins were dilated and thrombi composed of platelets and fibrin filled the lumen (Fig 17) The amount of fibrin in the thrombi were greater than at 10 minutes after the injection of ellagic acid The endothelium underneath these thrombi was often lacking (Fig 17) The myometrium seemed to be well preserved but often a large number of erythrocytes were found between the smooth muscle cells adjacent to myometrial vessels which contained thrombi

In the cases of partial necrosis only the area around the central artery was affected The adjacent lacunae of the giant cell layer were dilated and contained erythrocytes and extensive platelet fibrin masses The peripheral lacunae of the giant cell layer were not occluded In the lacunae of the roof dense platelet aggregates and platelet fibrin masses were found

In 20 placentas necrotic areas were not observed Platelet fibrin masses were occasionally found in the lacunae of the roof and in the lacunae of the giant cell layer and the amount of fibrin was greater than in the animals killed 3 and 10 minutes after ellagic acid injection The number of platelet aggregates in the tubular area and mural fibrin thrombi in lacunae of the roof were approximately the same as in placentas from animals killed 3 and 10 minutes after ellagic acid injection and in animals injected with buffered saline The same referred to thrombi in the uterine veins

Sacrifice at 10 Minutes and 24 Hours after Injection of Heparin and Ellagic Acid

When heparin was given prior to the injection of ellagic acid the formation of new platelet fibrin masses was prevented and their number was about the same as in untreated animals The occurrence of dense platelet aggregates and fibrin thrombi

the same as in untreated animals and animals treated with buffered saline

Electron Microscopy of Placentas 10 Minutes after Injection of Ellagic Acid or Buffered Saline

After ellagic acid injection densely packed platelets were seen in the lacunae of the roof and in the lacunae of the giant cell layer (Fig 18) In the periphery of the aggregates the platelets had lost their organelles More centrally in the aggregates the platelets had retained most of their organelles but showed varying degrees of pseudopod formation Granular masses of moderate electron density and fibrils suggestive of fibrin were encountered between the platelets and especially in the peripheral areas Occasionally red blood cells and leucocytes were seen between the platelets

Some of the platelets in the periphery of the aggregates appeared to be in close contact with microvilli of the syncytium

In the placenta taken 10 minutes after the injection of buffered saline only separate platelets were found in the lacunae of the roof and of the giant cell layer

Thrombosis in the Lungs Heart Liver and Kidneys

Dense platelet aggregates were often encountered in the small pulmonary vessels (Fig 19) in all animals examined both pregnant and non pregnant

Pregnant Mice

Platelet fibrin masses or fibrin thrombi were never observed outside the placenta and uterus in untreated animals and in animals given buffered saline (Table 4) In some of the pregnant mice injected with ellagic acid platelet fibrin masses were found in the pulmonary vessels (Fig 20) and the right ventricle of the heart most frequently at

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Figs 18–20

Fig 18 Electron micrograph of a lacuna of the giant cell layer ten minutes after injection of ellagic acid Aggregated platelets partly separated by granular masses and fibres suggestive of fibrin are filling the lumen In the periphery the platelets have lost their organelles and have decreased cytoplasmic electron density More centrally the platelets have retained their organelles although pseudopod formation is seen × 1[illegible] 000

Fig 19 Untreated pregnant mouse Pulmonary venule with a platelet aggregate HPS × 500

Fig 20 24 hours after injection of ellagic acid Platelet fibrin mass in pulmonary vein HPS × 75

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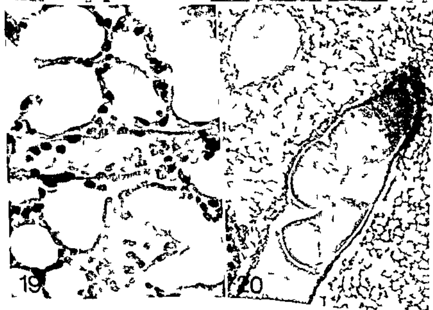


TABLE 4

The Number of Pregnant Animals in which Platelet Fibrin Masses were found in Lungs Heart Liver and Kidneys

Injection given	Time of sacrifice after injection	No of animals	Lungs	Heart	Liver	Kidneys
Ellagic acid	3 min	10	2	0	0	0
Ellagic acid	10 min	10	2	0	0	0
Ellagic acid	24 hrs	10	6	2	1	1
Untreated animals and animals treated with buffered saline		25	0	0	0	0
Ellagic acid and heparin		10	0	0	0	0

For subdivision of the group see Table 1

24 hours after the injection of ellagic acid (Table 4) Platelet fibrin masses were seen only in the kidney of one animal and in the liver of another animal

In the pregnant mice in which heparin was given before the injection of ellagic acid no platelet fibrin masses were observed outside uterus and placenta

Non Pregnant Mice

No platelet fibrin masses were seen in the organs of any of the 20 *non pregnant mice that were untreated or injected with buffered saline* In the 20 non pregnant animals injected with ellagic acid platelet fibrin masses were observed in the right ventricle of the heart of one animal sacrificed 10 minutes after the injection of ellagic acid No other platelet fibrin masses were observed in these animals in the heart lungs kidneys or liver

Bleeding Time and Clotting Time after Injection of Ellagic acid

Table 5 shows the effect of pregnancy and ellagic acid on bleeding and whole blood clotting times There was no significant difference in bleeding time between non pregnant and pregnant mice Nor did the injection of ellagic acid significantly alter bleeding time in either pregnant or non pregnant animals Whole blood clotting time was not significantly different in untreated non pregnant and pregnant animals The injection of ellagic acid significantly shortened whole blood clotting time in pregnant animals (Wilcoxon test pregnant untreated + pregnant buffer vs pregnant ellagic acid $p < 0.01$) In non pregnant animals the injection of ellagic acid also gave a reduced median whole blood clotting time but the difference is not statistically significant

TABLE 5

The Effect of Ellagic Acid on the Bleeding and Clotting Times in Non Pregnant and 16 Days Pregnant Mice

	No of animals	Bleeding time (secs)		Whole blood clotting time (secs)	
		Median	Range	Median	Range
Non pregnant Untreated	10	210	110–>600	90	30–190
Non pregnant Buffered saline	10	145	120–>600	52 5	35–260
Non pregnant Ellagic acid	10	145	90– 200	45	30–120
Pregnant Untreated	10	190	140–>600	77 5	60–210
Pregnant Buffered saline	10	225	190–>600	75	40–130
Pregnant Ellagic acid	10	170	120–>600	45	30–120

TABLE 6

Fibrinogen Determination in Mice
The Individual Highest Dilution Titre with Precipitate

	No of animals	Titre		
		1/80	1/160	1/320
Non pregnant	10	1	9	0
Pregnant	10	0	8	2
Pregnant 10 minutes after ellagic acid	10	0	8	2
Pregnant 24 hours after ellagic acid	10	0	7	3

Fibrinogen Determination

Table 6 shows fibrinogen values in non pregnant and pregnant mice and in pregnant mice given ellagic acid There was no obvious difference between the groups The injection of ellagic acid did not result in a fall of the fibrinogen level

DISCUSSION

Light microscopy of the mouse placenta showed at day 16 of gestation platelet aggregates platelet fibrin masses and fibrin thrombi in the maternal blood channels Thus as in the normal human placenta (17) there is a tendency to thrombosis in the mouse placenta

Dense platelet aggregates were found in nearly all areas of the maternal blood channels in the placenta and in the uterine veins the fibrin thrombi were especially located to the lacunae and the uterine veins

Occasionally non occluding dense platelet aggregates in the lacunae and in the uterine veins appeared to be in contact with the vascular lining These aggregates could represent the initial stage in the development of fibrin thrombi (10 13) Platelet fibrin masses in contact with the vascular lining and fibrin mural thrombi with and *without persisting identifiable platelet masses are probably the later* stages The same stages in the development of fibrin thrombi could be recognized in the human placenta (17) Some of the mural thrombi were entirely or partly composed of a structureless or finely granular material In the previous study of the human placenta evidence was presented which indicated that these hyaline deposits were composed of aged fibrin (16 17)

Most of the dense platelet aggregates in the lacunae and in the uterine veins appeared to be floating free These aggregates could be emboli from platelet aggregates attached to upstream placental structures they could be cross sections of pendulating mural platelet aggregates or they could have been formed in flowing blood (10) as in Chandler s loop (4) Considering the small number of aggregates in contact with the vascular lining it is likely that a great proportion of them are formed in the flowing blood

Even if the dense platelet aggregates are formed in flowing blood they may secondarily give rise to mural platelet thrombi and later to mural fibrin thrombi *Jorgensen et al* (12) showed that platelet aggregation in the microcirculation may give rise to focal vascular damage and mural thrombi The morphological evidence of vascular damage included rupture of the peripheral membrane of the endothelial cells discharge of the cellular organelles and later disappearance of the cells underneath the mural thrombi Also in the present study the lining cells underlying the mural dense platelet aggregates and platelet fibrin masses occasionally showed signs of alterations Underneath the mural fibrin thrombi the lining cells were often lacking These changes could well be the result of a secondary focal injury in association with primary platelet aggregation in flowing blood On the other hand the signs of damage could also be a morphological expression of a primary focal alteration in the vascular lining leading to mural thrombosis However altered lining cells not associated with thrombotic materials were rarely seen

In a previous paper (17) we summarized observations which gave strong evidence for the view that platelet massing in vivo is largely independent of the exposed surface and is rather governed by the flow pattern From the structure of the human placenta we deduced that marked irregularities in flow were bound to occur (17) Similar reflections can be done in the case of the mouse placenta When *the blood enters the lacunae of the roof from* the narrow central artery the flow must be split and the direction abruptly changed This will necessarily entail the formation of a lot of eddies In the tubular

area the flow probably resumes a more linear pattern whereas irregularities of the flow may again occur in the wide lacunae of the giant cell layer and in the uterine veins

Mural fibrin thrombi were found in the areas with assumed flow irregularities i e in the lacunae and in the uterine veins In the lacunae of the roof the thrombi were particularly frequent in curved parts or small pockets where eddy formation is bound to be marked In the tubular area where the flow may be more linear no distinct fibrin thrombi were seen The dense platelet aggregates seen in this part of the placenta could be emboli from the platelet masses found in the lacunae of the roof

An increased local stimulation of the coagulation through release of tissue thromboplastin could accelerate thrombus formation In the human placenta and decidua (23) the content of tissue thromboplastin is very high However the external coagulation system plays a minor role for the stabilization of platelet aggregates (9 11)

In conclusion dense platelet aggregates and mural thrombi form in the maternal blood spaces of the normal mouse placenta as in the human placenta It is likely that disturbances of flow are important for the formation of the thrombotic material Alterations or disappearance of lining cells underneath some of the mural thrombi may be a phenomenon secondary to the thrombosis Release of tissue thromboplastin locally could accelerate the thrombus formation but is probably of minor importance

Increased coagulation activity of the maternal blood in general is also considered to promote thrombus formation in the normal human placenta (for review see 17) However the relatively crude method for fibrinogen determination (7 24) presently employed did not reveal obvious differences between pregnant and non pregnant mice and the whole blood clotting times were similar in the two groups Platelet fibrin masses or fibrin thrombi were not observed in organs outside placenta in animals not given ellagic acid Thus by these parameters evidence of a generalized increased tendency towards thrombosis could not be found in the pregnant mouse

A local and/or generalized diminished fibrinolytic activity may also play a role suggested by the persistence of fibrin in the placental thrombi and the lack of morphological signs of lysis *Phillips et al* (19) injected thrombin into pregnant and non pregnant rats and found that thrombi tended to remain longer in renal glomeruli in pregnant animals compared with non pregnant this may be due to diminished fibrinolytic activity during gestation

Like thrombi in the human placenta (17) the thrombi in the mouse placenta did not undergo organization in the usual way Leucocytes were rarely attracted by the thrombi in the placenta in contrast to thrombi in the uterine veins where leucocytes were often seen within them Occasionally

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ULTRASTRUCTURE OF GRANULAR CELL MYOBLASTOMA

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Granular cell myoblastomas are among the controversial tumors Not only has there been disagreement about their origin but also about whether the entity represents a neoplasm or a histiocytic response In this electron microscopic study of three granular cell myoblastomas support for the neurogenic origin was obtained by the finding of tumor cells with distinct basement membranes containing cell inclusions of three different kinds and varying amounts of unmyelinated axons The three cell inclusions were The osmiophilic concentric lamellae the homogeneous lighter bodies and the aggregates of tubular filaments The study demonstrated that the osmiophilic concentric lamellae and the homogeneous lighter bodies take origin from degenerating mitochondria within the unmyelinated axons and the aggregates of tubular filaments appear to take origin from the neurofilament within the unmyelinated axons

It is evident from the twenty different names attached to granular cell myoblastoma that there is considerable controversy in regard to the nature of the lesion and its tissue of origin *Abrikossoff* in 1926 was the first to describe the classical histopathology of five previously uncharacterized tumors and coined the name Granular Cell Myoblastoma as he believed th s tumors to develop from undifferentiated striated muscle cells This myogenic concept attracted limited support Histocytic derivation has been favored by a number of investigators (*Leroux & Delarue* 1939 and *A opardi* 1956) They did not consider the lesions to be true neoplasms but rather histiocytic accumulation of lipid and protein A neurogenic theory of origin initiated by Feyrter in 1935 is supported by histochemical and in recent years electron microscopic studies

This work presents the ultrastructural features of three granular cell myoblastoma demonstrating that the granular cell contains three different cell inclusions and that all these inclusions take origin within the unmyelinated axon

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This paper was presented at the 59th Annual Meeting of the International Academy of Pathology in March 1970 at St Louis

MATERIAL AND METHODS

The first patient was a 58 year old female who had a subcutaneous tumor present on the medial aspect of the left arm for two and a half months Grossly the tumor was firm and white with a diameter of three centimeters Figure 1 shows a representative area with the granular cells arranged in long sheets and whorls surrounded by a few perivascular histiocytes and collagen

The second patient was a 42 year old female admitted with diarrhea A barium enema demonstrated a cecal polyp This was surgically removed In the submucosa there was a white firm circular mass with a diameter of 7 mm The pati nt had had another granular cell myoblastoma removed from the stomach six years previously

The third patient was a 50 year old female who died of sepsis and gangrene secondary to infarction of the bowel At autopsy a two and a half centimeter submucosal yellow white tumor was found in the stomach During a laparotomy five years



Fig 1 Granular cell myoblastoma with the tumor cells arranged in long sheaths and whorls surrounded by collagen and a few histiocytes (PAS 45 ×)

previously a small granular cell myoblastoma was removed from the same area of the stomach

The tissue from the first patient was fixed immediately in 3 per cent gluteraldehyde The tissue from the second and third patients was first fixed in (buffered) 10 per cent formalin for several days The tissue from all patients were postfixed in osmium tetroxide dehydrated in graded ethanol and embedded in Maraglas Ultra thin sections were stained with uranyl acetate and lead citrate and viewed under a Jeol Jem 50 and Siemens Elmiskop 1 electron microscope

RESULTS

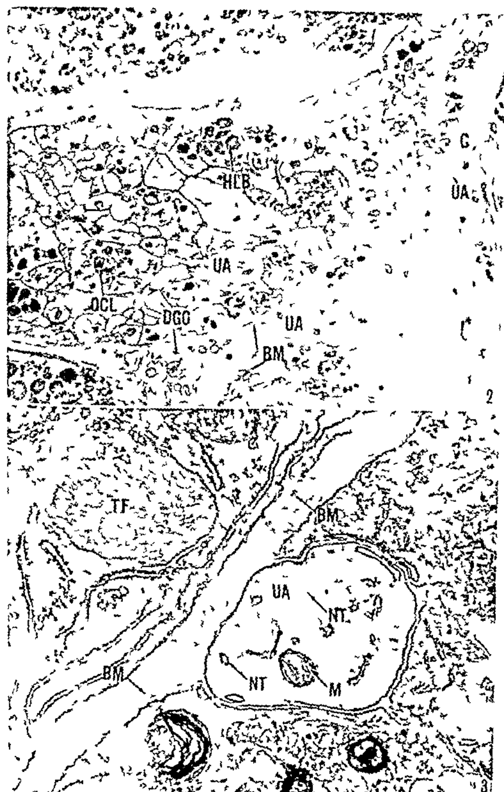
Electron microscopy revealed all the tumors to have a consistent pattern The tumor cells were either single or arranged in compact groups or whorls surrounded by abundant collagen and a few histiocytes Each of the granular cells was surrounded by distinc basement membranes Unmyelinated axons were present in most of the granular cells (Fig 2) In the best preserved axons neurotubules, neurofilaments and mitochondria were identifiable (Fig 3)

Cell inclusions of three different kinds were encountered in the cytoplasm of the granular cells Two were oval to round granules ranging in size from 0 5 to 3 microns in diameter One kind of granule contained osmiophilic concentric lamellae, the so called myelin figures These lamellae were arranged concentrically or appeared to have several centers (Figs 4 and 5) The other kind of granules was less osmiophilic but more homogeneous in staining characteristics often irregular in shape with varying substructures present (Fig 5) Both types of granules were partially or completely membranebound A third type of cell inclusion consisted of aggregates of tubular filaments again partially or completely membranebound These aggregates were not so frequent as the two previously described granules but were found in all the tumors examined They were usually found in the periphery of the granular cells and were often in close contact with the cell membrane (Figs 3 and 7)

In multiple sections examined one gained the impression that both kinds of granules originated from degenerating mitochondria within the unmyelinated axons Smooth tran

Fig 2 Central granular cell surrounded by portions of four other granular cells all with distinct basement membranes The two different granules The *osmiophilic concentric lamellae (OCL)* The homogeneous lighter bodies (HLB) and many unmyelinated axons (UA) are seen A degenerating granular cell (DGC) is below the central cell Collagen (C) is found to interdigitate with the base ment membrane and run parallel to this structure (1 250 ×)

Fig 3 Note the border of two granular cells with distinct basement membranes (BM) To the righ is an unmyelinated axon (UA) containing neuro tubules (NT) neurofilaments (NF) and one mito chondrion (M) To the left is an aggregate of tubular filament (TF) with prominent rough en doplasmic reticulum (20 000 ×)



C
UA
HLB
UA
OCL
DGO
UA
BM
2
TF
BM
UA
NT
NT
M
BM
3

sitions from relatively normal to the osmiophilic concentric lamellae or lighter homogeneous bodies were found (Figs 6)

The aggregates of tubular filaments seemed to take origin from the neurofilaments of the unmyelinated axons and fuse to form larger and larger aggregates close to the cell membrane (Fig 7) In the triangular to spindle shaped histiocytes, large aggregates of tubular filaments were noted and some histiocytes, especially the ones in perivascular position, were completely filled with this material (Fig 8) Many of the granular cells revealed prominent rough endoplasmic reticulum (Figs 3 and 7) and well formed Golgi complexes consisting of numerous vacuoles and flat saccules (Fig 9)

In some of the granular cells pinocytotic vesicles were noted Collagen was very prominent between the granular cells and was found to interdigitate with the basement membrane and, run parallel with this structure (Fig 2) No mitoses were noted Myofibrils and myelinated axons were not seen in any of the granular cell myoblastomas examined

Comparing different areas of the three tumors, and by making montages it became evident that the granular cells were in different stages of development Some contained only a few granules, and many unmyelinated axons while others were filled with the different cell inclusions but had only a few identifiable unmyelinated axons Cells in the same stage would generally be grouped together Viruses or viruslike particles were not observed There were irregular clear cells between the granular cells (Fig 2) The most prominent features of these cells were a large irregular nucleus very scanty cytoplasm with rough endoplasmic reticulum mitochondria and an occasional granule or aggregate of tubular filaments These cells were regarded as degenerating cells and could represent old burned-out granular cells or histiocytes

DISCUSSION

Several investigators have described the fine structure of granular cell myoblastoma *Hausken & Langer* in 1962 concluded that granular cells were the result of an irreversible metabolic disturbance, which could take place in many different cells, such as the connective tissue cells Schwann cells or striated muscle cells These authors described three different cell inclusions, two forms of granules one containing homogeneous slightly osmiophilic material, the other concentric membranes and finally in the histiocytes parallel bundles of filaments

Fischer & Wechsler in 1962 did electron microscopy as well as a variety of histochemical studies of five examples of granular cell myoblastoma and concluded a derivation from Schwann cells These authors favored a histiocytic response rather than a neoplasm They also compared similarities between granular cell myoblastoma cells and changes in Schwann cells after mechanical injury to the nerve They found viruslike particles consistently in the tumor cells

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Fig 4 Multicentric osmiophilic lamellae suggestive of mitochondrial origin are shown (50 000 ×)

Fig 5 Two granular cells with both types of granules The osmiophilic concentric lamellae (OCL) and the homogeneous lighter bodies (HLB) with varying substructures (10 000 ×)

Fig 6 The edge of a granular cell with distinct basement membrane (BM) and multiple unmyelinated axons Notice the transition of mitochondria from relative normal (M) to homogeneous lighter bodies (HLB) with remnants of crista (16 250 ×)

Fig 7 Illustration of the border of two granular cells In the cell to the right several aggregates of tubular filaments (TF) can be seen in close contact with the cell membrane The tubular filaments seem to take origin from the neurofilaments (Arrow) and fuse to form larger and larger aggregates partly membrane bound (12 500 ×)

Fig 8 Histiocyte loaded with aggregates of tubular filaments (15 000 ×)

Fig 9 Granular cell with well developed Golgi complexes (GC) consisting of numerous vacuoles and flat saccules (7 500 ×)


8
GC

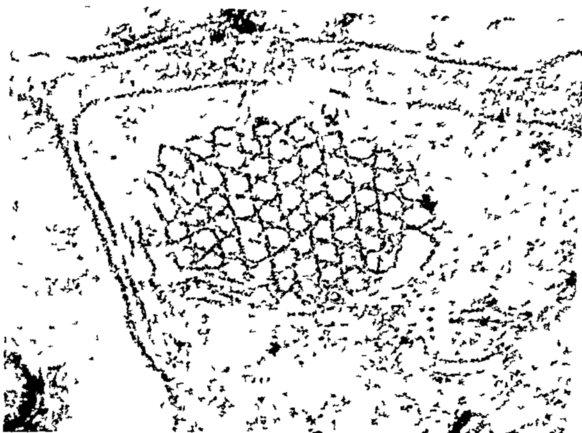


Fig 1 a Honeycomb structure in unmyelinated axon Note the tubules packed in a hexagonal parallel array (Magnification 30 000 ×)

tubules or neurofilaments were established (Fig 1 a)

By review of the literature a few similar structures were found This honeycomb arrangement of tubules has been described in chief cells from the gastric mucosa of hibernating bats (*Ito & Winchester* 1963) The authors regarded the structures as a previous undescribed differentiation of the endoplasmic reticulum studded with six pairs of ribosomes around the circumference of each tubule More recently similar parallel aggregates of tubules were noted in the myelinated and unmyelinated axons from the granular cell layer in normal and jimpy mice (*Hirano et al* 1969) and in the neurons of the cerebellum in the cat (*Morales & Duncan* 1966) Both studies indicated an origin from endoplasmic reticulum

The functional significanse or these honeycomb structures remain obscure It is of considerable interest however to find this structure in a Granular Cell Myoblastoma which recently has received further evidence for its nervous origin (*Garancis et al Carstens* 1970)

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PERFUSION FIXATION WITH GLUTARALDEHYDE FOR ELECTRONMICROSCOPY OF HUMAN THYROID TUMOURS

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A method has been worked out to perfuse a thyroid lobe containing a thyroid tumour immediately after it has been removed from the organism. The surgical technique and the fixation method are described The preservation of the thyroid tissue that was achieved with the described method is of adequate quality and comparable with results obtained in experimental animals The method has so far been used in 30 cases of which 4 are represented by micrographs in this preliminary communication

In immersion fixation of thyroid tissue for electronmicroscopy one generally gets considerably better preservation in experimental animals than in man This depends on difficulties of getting well vascularized tissue and on the slow penetration of fixing fluid due to the large amounts of connective tissue In human experiments the time from the resection of the tissue until fixation can take place is relatively long In order to study the ultrastructure of human thyroid tumours a method has been worked out to supply the fixation medium to the tumour via the inferior or superior thyroid artery

METHODS

1 Surgical Method

At the operation the thyroid lobe containing the tumour is dissected free very carefully to avoid haemorrhage and laceration of the tissue The arterial circulation is maintained during the whole operation via the artery one intends to use for perfusion Right up to the time the tumour is removed a sufficient venous outflow must be present so that stasis is avoided In the last stage of the operation the lobe is resected in the isthmus region without any use of artery forceps so that bleeding can take place via free tissue surfaces The last remaining vein is divided but not ligated on the preparation side A cannula is inserted in the artery which is ligated and the lobe removed quickly Perfusion is begun immediately via the cannula inserted in the artery

Fixing Technique

The lobe is slowly perfused with 1 5 per cent or 3 0 per cent glutaraldehyde in Tyrodes solution at pH 7 3–7 4 In order to facilitate the perfusion a *free venous outflow from the lobe must be present* This outflow takes place through the resection surface and through veins which during the operation were ligated but now are opened by cutting the ligatures In general perfusion takes place with 60–80 ml of glutaraldehyde at a flow speed of about 0 2 ml per second After excision of a sufficiently large piece of tissue typical of the tumour for electronmicroscopy the tumour is sent for patho-anatomical investigation which is not made more difficult by glutaraldehyde fixation The piece of tissue intended for electron microscopy is divided into small pieces of tissue about 1 mm^3 which thereafter are transferred to cold

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1 per cent osmium tetroxide solution, which is blood isotonic with pH 7 2 After fixation in osmium for 2 hours, dehydration and embedding in Epon 812 takes place

RESULTS

So far the method has been used in 30 cases of thyroid tumour

In judging the quality of preservation it was considered that the preservation of normal thyroid tissue of experimental animals, obtained by perfusion with glutaraldehyde followed by immersion in osmic acid satisfies high demands If judged on this basis fixation in all investigated cases has been satisfactory and in most of them very good The results can be summarized in the following way

1 Fixation is homogeneous over a large tissue area and does not vary from one cell group to another (Fig 1)

2 The intercellular spaces are not dilated (Figs 1 + 2)

3 The cells do not show any signs of swelling or shrinking and the cell membranes are intact (Figs 1 + 2)

4 The membranes of the endoplasmic reticulum are well preserved and furnished with numerous ribosomes (Fig 3) The sacculi vesicles and lysosomes of the Golgi apparatus as well as the mitochondria have intact membranes (Figs 3 + 4) No deformation of the organelles can be observed (Figs 2–4) The matrix of the cytoplasm has a homogeneous appearance with a relatively high electron optic density without any signs of vasculization or disaggregation (Figs 2–4) The nucleus



Fig 1 Mixed form of papillary and follicular thyroid carcinoma Intra as well as extra follicular cells are well fixed × 3 300



Fig Papillary thyroid carcinoma Nuclei with irregular contour and large clearly demarcated nucleoli Well visible cell boundaries small Golgi zones with flat sacculi the membranes of which have a parallel course Plentiful occurrence of small vesicles and lysosomes of varying size chiefly in the vicinity of the Golgi zones At the top of the micrograph a narrow follicle lumen (= f) × 14 000

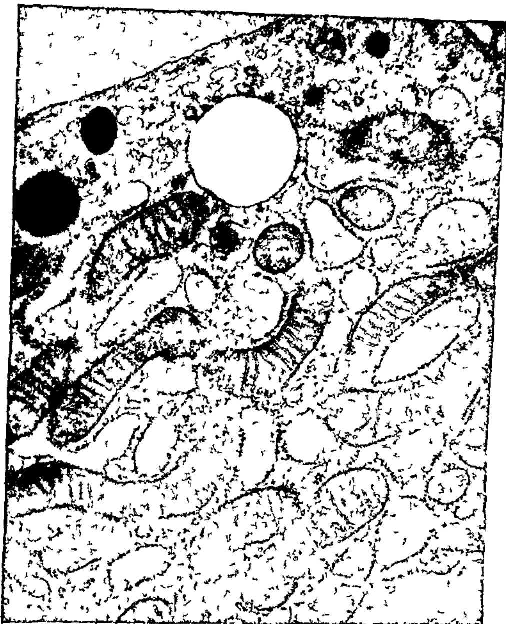


Fig 3 Inactive follicular thyroid adenoma Below to the right the basal membrane at the top left the apical plasmamembrane x 33 000

has a content of relatively high density, the *nucleoulus* is clearly *demarcated* and *the en*velope of the nucleus is well preserved (Fig 2)

DISCUSSION

At an international congress on thyroid cancer *in Lausanne* in 1968 stress was laid on the importance of electronmicroscopical studies of thyroid tumours in order to create a foundation *for uniform classification* and *better* understanding of the biological activity of thyroid tumours (1) Only few works which deal with the ultra structure of thyroid tumours have been published One reason for this is certainly that it is very difficult to obtain well preserved material with conventional technique (2)

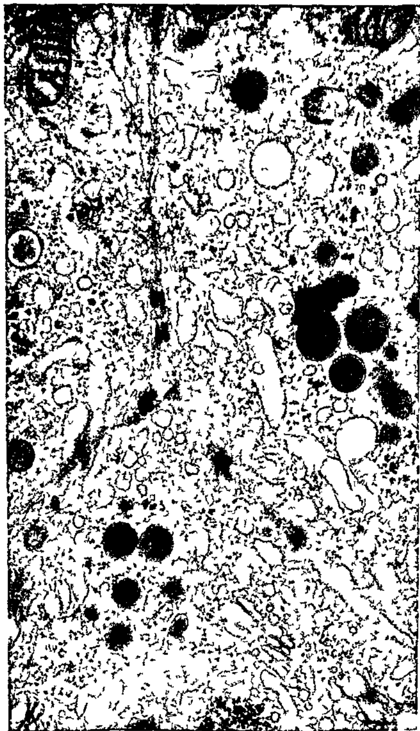


Fig 4 Follicular thyroid adenoma Three bordering epithelial cells close to one another Glycogen granules in all cells Top left a well preserved mitochondrion Golgi zone lysosomes and multivescular bodies and a large number of free ribosomes are observed × 32 000

In order to get good preservation of tissue it is necessary to have an intimate collaboration between surgeon and electronmicroscopist The specimen must be taken while the patient is on the operation table with the least possible loss of time Serious intra-cellular injury can be caused by mechanical influence or anoxia Fixation must take place quickly after removal of the tumour and the fixative should be able to penetrate rapidly into the cells

In animal experiments carried out in recent years laboratory animals have been performed *in vivo* with glutaraldehyde via the aorta and it has been achieved to have the tissue fixed at the moment of death (3 4 5) With the method described here and in immediate connection with the operation the tissue is perfused via an artery In general it takes between 30 and 120 seconds from the time the arterial flow to the lobe is stopped until the perfusion is begun With increased practice and skill this time can be reduced still further The method has been used for fixation of thyroid tumours but it can be used also for fixing other human tumours if the surgical procedure comprises resection of so large a portion of the organ that a sufficiently large artery is available for perfusion During the last year it has been possible to demonstrate that this method can be used to achieve well preserved parathyroid tissue in patients operated on for hyperparathyroidism

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RENAL TRANSPLANTATION IN RABBITS

II *Morphological Alterations in Autografts*

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Histopathological changes have been studied in 82 specimens from 52 autografts removed from 1 hour to 45 days after transplantation Discrete lymphocytic infiltrates were found in about 30 per cent of the autografts Swelling or proliferation of the endothelium in glomeruli and intrarenal vessels was found in about 25 per cent of the grafts, and exudative changes in glomeruli or vessels were seen in about 10 per cent of the autografts Glomerular microthrombosis occurred in 10 per cent of the grafts always in connection with recent vascular thrombosis In conclusion the same type of histopathological changes described as occurring in the acute *allograft* reaction may also be found in *autografts* The lesions in autografts however are inconstant and slight compared with those in allografts These observations indicate that the kidney graft possesses a reaction pattern which can be activated by non immunological as well as by immunological factors

The morphological changes in kidney auto grafts are generally discrete compared with the massive lesions in unmodified kidney allo grafts (1 2 3 4 10 11)

These changes however are interesting from a theoretical point of view because they illustrate the effect of non immunological factors on the kidney graft and form the base line necessary for the evaluation of the effect of immunological factors on the histopatho logic reaction in kidney allografts (*Lund & Myhre Jensen* 1970)

In the present study the frequency extent and nature of histopathologic changes in rab bit kidney autografts have been analysed semi quantitatively The first series of experiments was also used for practising the operative technique A second series of experiments in which the autografts were studied by repeated biopsies was therefore performed in order to complete the study

MATERIAL AND METHODS

Adult New Zealand white rabbits weighing 2–3 kg were used Rabbits were fed a common labo ratory diet plus fresh vegetables and allowed drink ing water ad libitum

Kidney transplantation was performed by end to side anastomosis between the renal vessels of the left kidney and the abdominal aorta and inferior vena cava and ureter was anastomosed to the bladder Warm ischaemia lasted 1–2 minutes the period of cold ischaemia averaged 45 min utes (range 30 to 60 minutes) The right kidney was not handled in order to minimize the uraemia caused by a decreased function of the kidney graft during the first days after transplantation

Thirty five kidney autografts removed within the first 24 hours after transplantation because of death of the rabbit or because of obvious technical failure are not included in the present material The kidney grafts reported here showed normal tension and a red colour after reestablishment of the circulation and produced urine before the ureterovesical anastomosis was performed Details of the operative technique postoperative care and

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TABLE 1 *Histopathological Alterations in 8? Specimens from 52 Kidney Autografts Removed 1 to 45 Days after Transplantation Biopsies are marked by*

Day	Rabbit no	Cell infilt	Prolif Changes glomer	Prolif Changes vessels	Exudat Changes glomer	Exudat Changes vessels	Thrombosis glomer	Thrombosis vessels	Necrosis
1/21	370	—	—	—	—	—	—	—	—
	372	—	—	—	—	—	—	—	—
	373	—	—	—	—	—	—	—	—
	374	—	—	—	—	—	—	—	—
	375	—	—	—	—	—	—	—	—
	376	—	—	—	—	—	—	—	—
	377	—	—	—	—	—	—	—	—
	379	+	—	—	—	—	—	—	—
1	102	—	+	—	—	—	—	+ +	+ + +
	103	—	—	—	—	—	—	—	—
	357	—	—	—	—	—	+ +	+ + +	+ + +
	370	—	—	—	—	—	—	—	—
	372	—	—	—	—	—	—	—	+ + +
	373	—	—	—	—	—	—	—	—
	374	—	—	—	—	—	—	—	—
	375	+	—	—	—	—	—	—	—
	376	—	—	—	—	—	—	—	—
	377	—	—		—	—	—	—	—
	379	—	—	—	—	—	—	—	—
	380	—	—	—	+	—	—	—	—
2	167	—	—	—	—	—	—	—	—
	168	—	—	—	—	—	—	—	—
	175	—	—	—	—	—	—	—	—
	323	—	—	—	—	—	—	+	+ + + +
	374	—	—	—	—	—	—	—	—
	375	—	—	—	—	—	—	—	—
	376	—	—	—	—	—	—	—	—
	377	—	—	—	—	—	—	—	—
	379	—	—	—	—	—	—	—	—
	380	—	—	—	—	—	—	+ + +	+ +
	381	—	—	—	—	—	—	—	—
3	370	—	—	—	—	—	—	+ + +	+ +
	355	—	—	—	—	—	—	+ +	+ +
	374	—	+	—	—	—	—	—	—
	375	—	—	—	—	—	—	—	—
	376	—	—	—	—	—	—	—	—
	377		—	—	—	—	—	—	—
	379	—	—	—	—	—	—	—	—
4	80	—	—	—	—	—	—	+ + +	+ + +
	109	—	—	—	—	—	—	+ + +	+ + + +
	335	—	+ + +	+	—	—	—	—	—
	359	+	+	—	—	—	—	—	—
	374	—	+ +	—	—	—	—	—	—
	375	—	—	—	—	—	—	—	—
	376	+	—	—	—	—	—	—	—
	377	—	—	—	—	—	—	—	—
5	50	—	—	—	—	—	+	+ +	+ + +
	101	—	—	—	—	—		+	+ + +
	182	—	—	—	—	—	—	—	+
	374	—	+	—	—	—	—	—	—
	375	—	—	—	—	—	—	—	—
	376	—	—	—	—	—	—	—	—
	377	—	—	—	—	—	—		

TABLE 1 *(cont.)*

Day	Rabbit no	Cell infilt	Prolif. Changes glomer	Prolif. Changes vessels	Exudat. Changes glomer	Exudat. Changes vessels	Thrombosis glomer	Thrombosis vessels	Necrosis
6	329	—	—	—	—	—	—	+ +	+ + +
	331	+	+ +	—	—	—	—	—	—
7	333	+	—	—	—	—	—	—	—
	363	+ + +	+ +	—	—	—	—	—	—
	365	+	—	—	—	—	—	—	—
	381	—	—	—	—	—	—	—	—
8	114	—	—	—	—	—	—	+ + +	+ + +
	337	+ + +	+ +	—	+	—	—	—	—
	353	+	—	—	—	—	—	—	—
9	351	—	—	—	—	—	—	+ + +	+ + +
10	315	—	—	—	—	—	—	+ + +	+ + + +
	318	+	—	—	+	—	—	—	—
11	313	+	—	—	—	—	—	—	—
12	113	+ +	+ +	+ +	+	+	+ + +	+ +	+ +
	316	—	—	—	—	—	—	+ + +	+ + +
13	40	+ +	+	+	+	+	+	+	+
	325	—	—	—	—	—	—	—	—
14	311	—	—	+	—	—	+	+ + +	+ + + +
	312	—	—	—	—	—	—	+ + +	+ + +
	327	+	—	—	—	—	—	—	—
	381'	—	—	—	—	—	—	—	—
21	93	—	+ + +	+	+	—	—	—	+
	172	+	+ + +	—	—	—	—	—	—
	381	+	—	—	—	—	—	—	—
30	180	—	—	—	—	—	—	+ + +	+ + + +
	181	—	—	—	—	—	—	+ + +	+ + + +
	183	—	—	—	—	—	—	+ +	+ + + +
45	169	+ +	+	—	—	—	—	—	—
	171	—	—	+	—	—	—	+ + +	+ + + +

Key for the semiquantitative evaluation

	Mononuclear cell infiltr	Glomerular changes	Vascular changes	Necrosis
+	small infiltrates	in less than 10 % of the glom	in a few vessels	patchy cortical necrosis
+ +	many or large perivascular	in 10–50 % of the glom	in many vessels	subcapsular zone
+ + +	diffuse infiltr	in more than 50 % of the glom	in almost all vessels	partial necrosis of cortex/medul
+ + + +				total necrosis

complications are described elsewhere (*Lund* 1970)

52 kidney grafts were removed for microscopical examination 1 to 45 days after transplantation (*I series*) From 10 autografts *performed during* a later period (II *series no 370–381*) 30 cortical wedge biopsies were obtained from 1 hour to 14 days after transplantation Five biopsies were taken *from each graft in 4 cases 3* biopsies in 2 cases and 1 biopsy in 4 cases

The wedge biopsies were performed under general anaesthesia (nembutal/N O/O) alternately from the upper and lower portions of the *graft After biopsy* the renal capsule was sutured with 6-0 atraumatic silk

Specimens of tissue for microscopy were *fixed in* 4 per cent buffered formaldehyde and *sections* of paraffin-embedded *material were stained* with haematoxylin eosin phosphotungstic acid haematoxylin Lendrum s stain for fibrin periodic acid Schiff Unna Pappenheim stain (methyl *green* pyronine) and picro-sirius *red Specimens* were examined microscopically *immediately* after preparation by one of us (BI) At a later date they were examined in a pool with other specimens from kidney allografts by OMJ, who had *not seen any of the* preparations before and who was not aware of the data *concerning* the specimens This evaluation was made semiquantitatively (Table 1)

RESULTS

As a rule renal autotransplants increased in weight during the first four or five days after transplantation and then remained stable at a level of about 50 *per* cent above pre operative weight

Results of the semiquantitative histological analysis of 52 autografts removed 1–45 days after transplantation *are shown in* Table 1 It appears that no histopathological changes except slight or moderate oedema were *found in* only 11 of the 52 autografts

Interstitial Cell Infiltration

Infiltration with mononuclear cells was found in 15 of the 52 autografts The cell infiltrates were located in close relationship to vessels *in the corticomedullary zone* (Fig 1) The number of cells varied from a few



Fig 1 Autograft no 331 removed 6 days after transplantation Infiltrate related to veins in the cortico medullary zone HE × 200

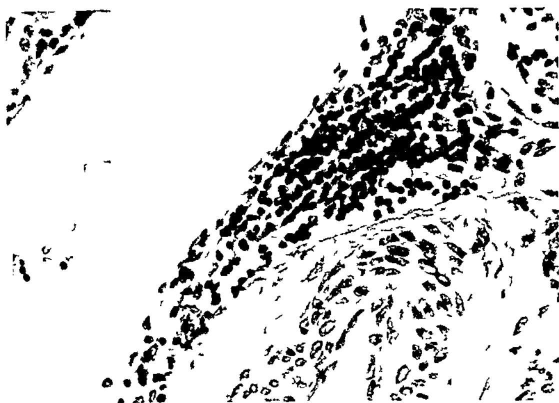


Fig 2 Autograft no 318 removed 10 days after transplantation Small infiltrate composed mainly of small lymphocytes HE × 400

cells to collars of cells around the vessels The main cellular constituent of the infiltrates was the small lymphocyte (Fig 2) whereas the larger lymphoblast like cell which is regularly seen in allografts dominated the infiltrates in only two cases (no 40 113)

Eosinophils were seen in most of the autografts as solitary cells or groups of 2 or 3 cells

Polymorphonuclear cells were seen in the demarcation zone around necrotic areas as well as in inflammatory exudates in glomeruli and vessels (see below)

Proliferative Changes in Glomeruli and Vessels

Proliferative glomerulitis characterized by swelling or proliferation of glomerular endothelial or mesangial cells was found in 12 of 52 autografts Definite proliferation of the glomerular cells was seen in 3 of these cases (Fig 3 and 4) Proliferative changes began to appear on the fourth day after transplantation (Fig 5) In the affected autografts proliferative glomerulitis was seen in 10–50 per cent of the glomeruli

Endarteritis characterized by swelling and/or proliferation of endothelial cells (Fig 6 and 7) was found in 5 autografts but in 3 cases the endarteritis was slight and affected only a few vessels Proliferative vascular changes were accompanied by proliferative glomerulitis in 3 of the 5 cases

Exudative Changes

Exudative glomerulitis characterized by exudation of polymorphonuclear leucocytes into the tuft of the glomeruli or into the glomerular capsular space was seen in 5 autografts When present however these changes appeared in less than 10 per cent of the glomeruli in each specimen

Exudative glomerulitis appeared 8 days after transplantation (except in no 380 where it was seen from the second day) and the

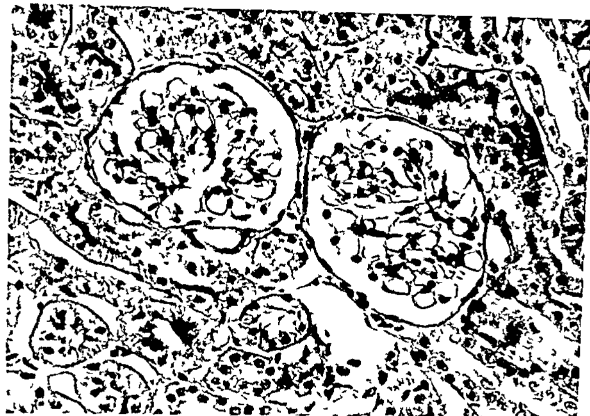


Fig 3 Control kidney Normal glomeruli PAS × 400

lesion was accompanied by proliferative glomerulitis in all cases but one

Vasculitis, characterized by an inflammatory exudate consisting of polymorphonuclear leucocytes and/or necrosis of vascular walls was found in 2 cases (12 and 13 days after transplantation) In both cases vasculitis was combined with proliferative changes glomerular microthrombosis thrombosis in other intrarenal vessels and necrosis

Thrombosis

Glomerular microthrombosis was found in 5 of 52 autografts Microthrombi were present in less than 10 per cent of the glomeruli in 3 cases and from about 10 per cent to over 50 per cent in two cases (Fig 8 and 9) When only a few glomeruli were affected the microthrombi appeared as one or two capillary fibrin plugs When several glomeruli were involved microthrombosis was usually seen in most of the capillary loops in each glomerulus

Thrombosis of the intrarenal blood vessels was found in 23 of 52 autografts Thrombosis was localized to veins in 8 of the cases whereas in the other cases thrombi were present in both veins and arteries

Thrombosis was accompanied by proliferative and exudative changes in glomeruli and vessels and by cellular infiltrates in 2 cases and in 2 other cases by endarteritis

Necrosis

Necrosis of renal tissue was classified according to the extent of the necrosis

Patchy cortical necrosis localized around one or a few interlobular arteries was found in 3 cases Proliferative and exudative changes in glomeruli and blood vessels accompanied the necrosis in 2 of the 3 cases Glomerular microthrombosis was found in 1 of the autografts

Subcapsular cortical necrosis affecting the peripheral one to two thirds of the cortex was found in four autografts One of these autografts showed lymphocytic infiltrates pro

liferative and exudative changes in glomeruli and vessels and glomerular microthrombosis in more than 50 per cent of the glomeruli (Fig 7–9)

Partial necrosis of the kidney affecting most of the cortex and outer medullary zone was found in 11 autografts A demarcation zone consisting of polymorphonuclear leucocytes was demonstrated in all cases but 2 In 2 of the autografts glomerular microthrombosis was seen

Necrosis of the entire kidney was found in 8 autografts

All the autografts except one showing subcapsular partial or total necrosis had demonstrable thrombosis of the intrarenal blood vessels whereas no thrombosis could be demonstrated in one of the autografts displaying partial necrosis and in 2 of the autografts with patchy cortical necrosis

Ten autotransplantations (nos 370–381) were performed at a later date than the other autotransplantations and from these 30 biopsies were obtained 1 hour to 14 days after transplantation The rate and severity of necrosis were less in this group of experiments in spite of the fact that these autografts were biopsied several times Interstitial lymphocytic infiltrates were found in only one biopsy from each of the three autografts showing cellular infiltrations indicating that the infiltrates were sporadic and therefore not present in every biopsy

In rabbit 374 no proliferative changes were seen in the 3 biopsies obtained during the first 2 days after transplantation but appeared in biopsies from the third and fourth days as well as in the specimens from the kidney graft which was removed on the fifth day

In rabbit no 380 (Table 1) proliferative and exudative glomerulitis was found in the biopsy taken 1 day after transplantation the specimen from the next day showed cortical necrosis

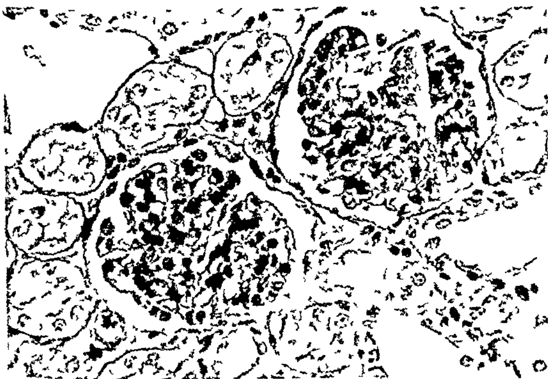


Fig 4 Autograft no 331 removed 6 days after transplantation Glomeruli with slight swelling and proliferation of endothelial and mesangial cells PAS × 400

4

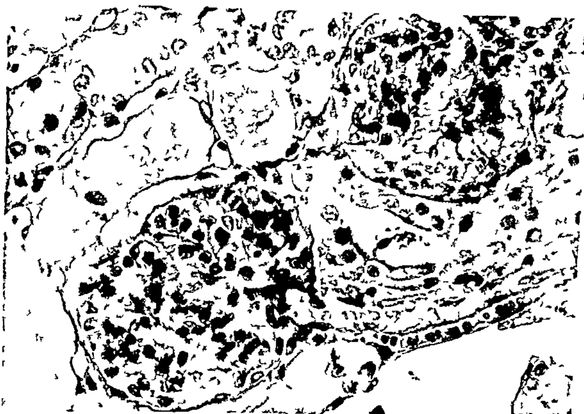


Fig 5 Autograft no 33ɔ removed 4 days after transplantation Pronounced proliferation of the cells of the glomerular tuft PAS × 400

DISCUSSION

Function of the graft Autotransplantation of a kidney does not always result in a functioning graft *Dempster* (1959) performed 143 kidney autotransplantations in dogs but only 50 grafts functioned for more than 3 days *Dempster* found that in most cases surgical complications such as disengagement of the renal artery thrombosis or breakdown of the suture line were responsible for the failure In some cases with non functioning kidneys however no obvious sign of surgical failure could be demonstrated, the grafts in some of these cases showed haemorrhagic necrosis and in other cases anuria developed shortly after reestablishment of the circulation without the development of haemorrhagic necrosis of the graft

Most of the later reports mention only functioning autografts (1 2, 4 10 11) *Porter* (1966) described the changes in functioning autografts as consisting of tubular degeneration minimal cell infiltration and an increase in weight the latter being ascribed to functional hypertrophy

The function of kidney grafts was not studied in the present experiments One of the rabbits own kidneys was left untouched in order to avoid any influence on the histological pattern caused by severe renal insufficiency The production of urine could not be followed by simple collection in our model and attempts of collecting urine through uretero cutaneous anastomosis failed in all cases (*Lund* 1970)

Lymphocytic infiltration and endothelial changes The present study has shown that the histopathological changes described in association with the allograft reaction may also be found in some autografts The changes in autografts however are inconstant and discrete compared with those in allografts

Interstitial infiltration by lymphocytes was

rare and the infiltrates were few and small during the first five days after transplantation. In autografts removed later than the fifth day, however, cell infiltrates were present in almost all cases not demonstrating necrosis. This relationship of the lymphocytic infiltration to time after transplantation demonstrates that this infiltration is related to transplantation rather than being an accidental finding which was present in the kidneys before operation.

The main cellular constituent of the infiltrates was the small lymphocyte in all cases but 2. These 2 cases in which larger lymphocytes dominated were the only ones with both cell infiltration and cortical necrosis. *Mahabir et al.* (1969) concluded from studies on the allograft reaction in kidney transplantations between two strains of highly inbred rats with only minor antigenic difference, that *small* lymphocytes did no harm to the grafts, whereas *large* lymphocytes were associated with damage. It is worth mentioning that cellular infiltrates in the acute *allo*graft reaction in outbred rabbits also consist mainly of small lymphocytes during the first 4 to 5 days after transplantation (*Lund & Myhre Jensen* 1970).

The localization of lymphocytic infiltrates is the same as in kidney allografts: near the vessels at the corticomedullary junction. In allografts, however, infiltrates appear as early as the first and second day.

In about half of the cases showing lymphocytic infiltration, endothelial changes were found (Table 1) and these changes appeared simultaneously with the infiltrates. The demonstration of glomerular and vascular changes resembling those seen in allografts suggests that the endothelial cells of the kidney react to some extent uniformly to non-immunological and immunological stimulation. The glomerular endothelial and mesangial cells seem to react to stimulation earlier than the vascular endothelium. The cause of

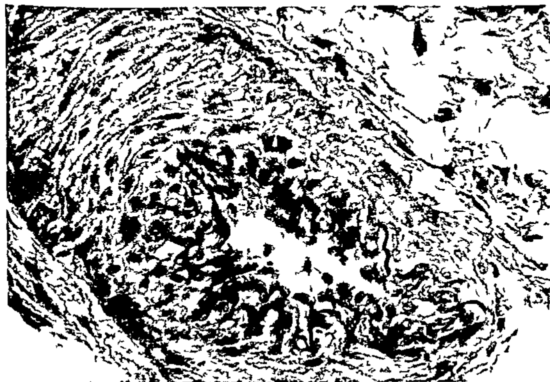


Fig. 6. Same autograft as Fig. 5. Moderate proliferation of the endothelial cells of an interlobular artery. PAS × 400.

lymphocytic infiltration and endothelial damage similar to those found in the allograft but more discrete is not clear. Conditions which could influence the kidney in autotransplantation are direct mechanical trauma, vascular spasms elicited by the operative procedures, low temperature and ischaemia.

Thrombosis and necrosis The correlation between necrosis and thrombosis of the intrarenal vessels is close (Table 1). Only 2 grafts (no 182 and 93) had patchy cortical necrosis without thrombosis of blood vessels or glomeruli. And all the grafts with thrombosis showed some type of necrosis. The high degree of correlation does not necessarily indicate a casual relationship. Both might be secondary to some other factor, for instance severe persistent vascular spasm. Serial biopsies from normal kidneys made ischaemic by ligation of blood vessels indicated that thrombosis of the intrarenal vessels appeared simultaneously with the first signs of necrosis and that no sign of necrosis could be demonstrated in biopsies within 24 hours after ligation (unpublished observation). It is worth mentioning that glomerular microthrombosis was seen in only 5 out of 26 autografts showing necrosis and thrombosis of the intrarenal vessels. In 2 of these (113 and 40) we found endothelial changes in the vessels and glomeruli which might have precipitated the microthrombosis. Glomerular microthrombosis was not demonstrated in the afore mentioned experiment where ligation of blood vessels was performed in normal non transplanted kidneys.

The greater frequency of graft necrosis in the first transplantations compared with that in those performed later at a time when transplantation had become routine illustrates the significance of the operative technique.

The various reaction patterns in the group of autografts as a whole may be due to

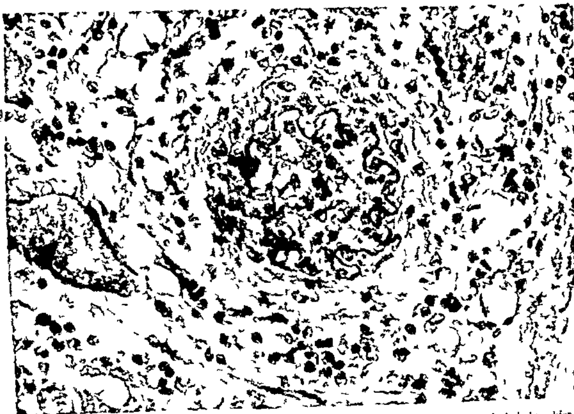


Fig 7 Autograft no 113 removed 12 days after transplantation. Artery with severe endothelial proliferation. Diffuse perivascular infiltration with large lymphocytes. PAS × 400



Fig 8 Same autograft as Fig 7 Cortical necrosis with massive subcapsular zone of demarcation (upper right) Thrombosis of an interlobular artery and four glomeruli PTAH × 100

individual variations in response to stimuli (grafting) It was our general impression at operation that some kidneys were more prone than others to develop spasm in the intrarenal vessels during the early postoperative period In 1949 *Trueta et al* showed that spasm of the intrarenal vessels might be provoked by mechanical or chemical stimuli and that the spasm caused a redistribution of the intrarenal bloodflow from cortex to a corticomedullary pathway The total bloodflow and the intrarenal distribution of blood in autografts have been studied by sine wave electromagnetic flowmeter and xenon washout technique (*Retik et al* 1967 *Hollenberg et al* 1968 *Rosen et al* 1967 *Jackson et al* 1967) No changes were demonstrated in *well functioning* autografts but *Rosen et al* and *Jackson et al* found a redistribution of blood in cases of autograft failure

Conclusion The present study indicates that the kidney graft possesses a characteristic reaction pattern which can be activated by non immunological factors The difference between histopathological changes in autografts and allografts is quantitative rather than qualitative Registration of the changes in kidney autografts forms a valuable and necessary baseline for the interpretation of changes in kidney allografts

The study was aided by grants from the Danish State Research Foundation and I I Smidth & Co Foundation

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Fig 9 Detail of Fig 8 Thrombosis of the arteriole and the vascular pole of a glomerulus PTAH × 400

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RENAL TRANSPLANTATION IN RABBITS

III Morphological Alterations in Allografts

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The quantitative morphological alterations in kidney allografts removed 1 to 14 days after transplantation have been studied in 118 rabbits Infiltration by small lymphocytes gradually increased from the first day after transplantation These cells were later transformed into or followed by larger lymphocytes which dominated the infiltrates after the fourth or fifth day Swelling or proliferation of endothelial and mesangial cells in the glomeruli slowly became more marked in frequency and extent followed by proliferations of vascular endothelium and exudation of polymorphonuclear cells and fibrin in glomeruli and vessels As a rule proliferative changes were more widespread than exudative changes in the glomeruli and vessels Thrombosis and cortical necrosis were frequent In only two cases however was *massive glomerular microthrombosis present In the rabbit the renal allograft reaction differs* from renal autograft reaction less in qualitative respects than in quantitative *i e* by the severity and early appearance of lesions in the allograft

In a previous report we demonstrated in rabbits that most of the morphological changes which have been regarded as characteristic for the kidney allograft reaction can also be demonstrated in some autografts (*Lund & Myhre Jensen* 1970) In the present report a semiquantitative analysis of the extent and nature of the morphological changes in kidney allografts has been performed The results are compared with those from autografts in order to elucidate the influence of immunological factors on the allograft reaction The findings also provide the baseline necessary for the evaluation of the histopathological alterations in kidney allografts in recipients sensitized against the kidney donor before transplantation (*Lund & Myhre Jensen* 1970)

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MATERIAL AND METHODS

Randomly bred adult rabbits weighing between 2 and 3 kg were used Rabbits were fed a common laboratory diet with additional fresh vegetables and allowed drinking water ad libitum Donor and recipient were chosen from different strains of rabbits In 60 cases the recipient was a New Zealand white rabbit in 35 a Brown Lop-eared rabbit and in 23 cases a Black Alaska rabbit Kidney transplantation between rabbits of same sex was done in 95 cases female to male rabbit in 15 cases and male to female rabbit in 8 cases

Kidney transplantation was performed by end to-side anastomosis between the renal vessels of the left donor kidney and the abdominal aorta and the inferior vena cava of the recipient Warm ischaemia lasted 1–2 minutes the period of cold ischaemia averaged 40 minutes (range 30–55 minutes) One of the recipient s own kidneys remained untouched the other one was used as a donor kidney in another operation Forty of the recipients own untouched kidneys were removed simultaneously with the allograft and served as controls Graft function was not studied No immunosuppressive therapy was given The operative technique postoperative care and complications are described in another paper (*Lund* 1970)

TABLE 1 *(cont.)*

Day	Rabbit no	Cell infiltr	Prolif changes glom	Prolif changes vessels	Exudat changes glom	Exudat changes vessels	Thrombosis glom	Thrombosis vessels	Necrosis
4	78	+ +	+	—	—	—	—	—	—
	79	—	—	—	—	—	—	+ + +	+ + + +
	99	—	—	—	—	—	—	—	—
	190	+ +	+ +	+	—	—	—	—	—
	415	+ +	+ +	+ +	—	+ +	—	—	+
	416	+	—	—	—	—	—	—	—
	432	+ +	+	—	—	—	—	—	—
	433	+ +	+	—	—	—	—	—	—
	440	—	—	—	+ + +	+ + +	+ + +	+ + +	+ + +
	445	+ +	+ + +	—	—	—	—	—	—
5	44	+ +	+	+ +	—	—	—	—	—
	49	—	—	—	—	—	—	—	—
	66	+ +	+ + +	+ + +	+ +	+	+	+ +	+ + +
	86	—	+	—	+	—	—	+ + +	—
	105	+ +	+ + +	+ + +	+ + +	+ +	—	—	—
	128	+ + +	+ + +	+ + +	+ +	+ + +	+	+	—
	130	—	—	—	—	—	—	+ + +	+ +
	133	—	—	—	—	—	—	+ +	+ +
	402	—	—	—	—	—	—	+ +	+ + +
	403	+ +	+ +	—	+	—	—	—	—
	404	+ +	+ +	+	—	—	—	—	—
	406	+ +	+ + +	+ + +	+	+ +	—	+ +	+ + + +
	410	—	—	—	—	—	—	—	—
	442	+ +	+ +	+	—	—	—	—	—
6	74	—	—	—	—	—	—	—	+ + + +
	76	—	—	—	—	—	+	+ + +	+ + +
	108	—	—	—	—	—	—	+ +	+ + + +
	112	+ + +	+ + +	+ + +	—	+ +	—	—	—
	119	—	—	—	—	—	—	+	+ +
	121	—	—	—	—	—	+ + +	+ + +	+ + +
	150	+ +	+ + +	+ + +	+ +	+ + +	—	+	—
	151	+	—	—	—	—	—	+	+ + +
	153	+ +	+ + +	+	—	—	—	—	—
	196	+ +	+ +	+	+ +	+	+ +	+ + +	+ +
	197	+ + +	+ + +	+	+	+	—	—	—
	437	+ +	+	+	—	+ +	—	—	—
7	25	—	—	—	—	—	—	+ + +	+ +
	85	+	+ +	+ + +	+	+ +	+	+ + +	+ + +
	126	+ +	+ +	+	—	—	—	—	—
	154	+ +	+ +	+	—	—	—	—	—
	157	+ + +	+ + +	+ + +	+ +	+ +	—	—	—
	163	+	+	+	—	—	—	—	—
8	67	—	—	—	—	—	—	+ +	+ + + +
	190	—	—	—	—	—	+ +	+ + +	+ + + +
	122	+ + +	+ +	+	—	—	—	—	—
	134	+ + +	+ + +	+ + +	+	+ +	—	—	—
	135	—	—	—	—	—	—	+ + +	+ + + +
	137	+ + +	+ + +	+ + +	—	+ +	—	—	—
	138	—	—	—	—	—	—	—	+ + + +
	139	+ + +	+ + +	+ + +	+	+ + +	+	+ +	+
	141	+ +	+ + +	+ +	—	+	—	—	—

TABLE 1 *(cont)*

Day	Rabbit no	Cell infiltr	Prolif changes glom	Prolif changes vessels	Exudat changes glom	Exudat changes vessels	Thrombosis glom	Thrombosis vessels	Necrosis
9	21	+ + +	+ + +	+ + +	+ +	+ + +	—	—	—
	107	+ +	+ +	+	—	—	—	—	—
	140	—	—	—	—	—	—	+	+ + +
	143	+ +	+ +	+	—	—	—	—	—
	144	+ + +	+ +	+ + +	+ +	+ + +	+	+	+
	146	—	—	—	—	—	—	+ +	+ + + +
	149	+ + +	+ +	+	+ +	+	+	+ +	+
10	148	+ + +	+ + +	+	—	+ +	—	—	—
	156	+ + +	+	+ + +	—	+ +	—	—	—
	159	+ +	+ + +	+	—	—	—	—	—
	164	+ + +	+ + +	+ + +	+ +	+ + +	+	+ + +	—
	165	+ +	+ +	—	—	—	—	—	—
11	65	+ + +	+ + +	+ + +	+	+ +	+ +	+ + +	—
12	185	+ + +	+ +	+ +	+ +	+ + +	—	—	—
	186	+ + +	+ + +	+ + +	+ +	+ + +	—	—	+
	187	+ +	+ +	+ + +	+ + +	+ + +	—	+ +	+ + +
	189	—	—	—	—	—	—	+ +	+ + +
	192	+ +	+	+	—	—	—	—	—
	193	+	—	—	—	—	—	+ + +	+ + +
13	422	+ +	+ +	+ +	—	+ +	—	—	—
	423	+ + +	+ + +	+ + +	+ +	+ + +	—	—	—
	424	+	—	+ +	+	+ +	+	+ + +	+
	441	+ + +	+	+ + +	+ + +	+ + +	+	—	—
14	413	+ +	+ +	—	+ +	+ +	+	+ +	+ + +
	414	+ +	+ +	+ +	—	+ +	—	—	—
	417	+ +	+	—	—	—	—	—	—
	418	+ + +	+ + +	+ + +	+ +	+ + +	—	—	—

Key for the semiquantitative evaluation

	Mononuclear cell infiltr	Glomerular changes	Vascular changes	Necrosis
+	small infiltrates	in less than 10 % of the glom	in a few vessels	patchy cortical necrosis
+ +	many or large perivascular	in 10–50 % of the glom	in many vessels	subcapsular zone
+ + +	diffuse infiltr	in more than 50 % of the glom	in almost all vessels	partial necr of cortex/medul
+ + + +				total necrosis

TABLE 1 (cont.)

Day	Rabbit no	Cell infiltr	Prolif changes glom	Prolif changes vessels	Exudat changes glom	Exudat changes vessels	Thrombosis glom	Thrombosis vessels	Necrosis
4	78	+ +	+	—	—	—	—	—	—
	79	—	—	—	—	—	—	+ + +	+ + + +
	99	—	—	—	—	—	—	—	—
	190	+ +	+ +	+	—	—	—	—	—
	415	+ +	+ +	+ +	—	+ +	—	—	+
	416	+	—	—	—	—	—	—	—
	432	+ +	+	—	—	—	—	—	—
	433	+ +	+	—	—	—	—	—	—
	440	—	—	—	+ + +	+ + +	+ + +	+ + +	+ + +
	445	+ +	+ + +	—	—	—	—	—	—
5	44	+ +	+	+ +	—	—	—	—	—
	49	—	—	—	—	—	—	—	—
	66	+ +	+ + +	+ + +	+ +	+	+	+ +	+ + +
	86	—	+	—	+	—	—	+ + +	—
	105	+ +	+ + +	+ + +	+ + +	+ +	—	—	—
	128	+ + +	+ + +	+ + +	+ +	+ + +	+	+	—
	130	—	—	—	—	—	—	+ + +	+ +
	133	—	—	—	—	—	—	+ +	+ +
	40?	—	—	—	—	—	—	+ +	+ + +
	403	+ +	+ +	—	+	—	—	—	—
	404	+ +	+ +	+	—	—	—	—	—
	406	+ +	+ + +	+ + +	+	+ +	—	—	—
	410	—	—	—	—	—	—	+ +	+ + + +
	442	+ +	+ +	+	—	—	—	—	—
6	?4	—	—	—	—	—	—	—	+ + + +
	76	—	—	—	—	—	+	+ + +	+ + +
	108	—	—	—	—	—	—	+ +	+ + + +
	112	+ + +	+ + +	+ + +	—	+ +	—	—	—
	119	—	—	—	—	—	—	+	+ +
	121	—	—	—	—	—	+ + +	+ + +	+ + +
	150	+ +	+ + +	+ + +	+ +	+ + +	—	+	—
	151	+	—	—	—	—	—	+	+ + +
	153	+ +	+ + +	+	—	—	—	—	—
	196	+ +	+ +	+	+ +	+	+ +	+ + +	+ +
	197	+ + +	+ + +	+	+	+	—	—	—
	437	+ +	+	+	—	+ +	—	—	—
7	25	—	—	—	—	—	—	+ + +	+ +
	85	+	+ +	+ + +	+	+ +	+	+ + +	+ + +
	126	+ +	+ +	+	—	—	—	—	—
	154	+ +	+ +	+	—	—	—	—	—
	157	+ + +	+ + +	+ + +	+ +	+ +	—	—	—
	163	+	+	+	—	—	—	—	—
8	67	—	—	—	—	—	—	+ +	+ + + +
	120	—	—	—	—	—	+ +	+ + +	+ + + +
	122	+ + +	+ +	+	—	—	—	—	—
	134	+ + +	+ + +	+ + +	+	+ +	—	+ + +	+ + + +
	135	—	—	—	—	—	—	—	—
	137	+ + +	+ + +	+ + +	—	+ +	—	—	+ + + +
	138	—	—	—	—	—	—	—	—
	139	+ + +	+ + +	+ + +	+	+ + +	+	+ +	+
	141	+ +	+ + +	+ +	—	+	—	—	—

TABLE 1 (cont

Day	Rabbit no	Cell infiltr	Prolif changes glom	Prolif changes vessels	Exud changes glom	Exud changes vessels	Thrombosis glom	Thrombosis vessels	Necrosis
	21	+ + +	+ + +	+ + –	+			—	—
	107	+ +	+ +	+	—			—	—
	140	—	—	—	—			–	– – –
9	143	+ +	+ +	+	—	—		—	—
	144	+ + +	+ +	+ + –	– –			–	–
	146	—	—	—	—	—		–	– – –
	149	+ + +	+ +	–	–			–	
	148	+ + +	+ + +	–	—				
	156	+ + +	+	+ – –	—				
10	159	+ +	+ + +	+	—				
	164	+ + +	+ + +	+ – –	– +	–			—
	165	+ +	+ +	—	—				
11	65	+ + +	+ + +	+ + +	+	+ +			
	185	+ + +	+ +	+ +	+ +	+ +			
	186	+ + +	+ + +	+ + +	+ +	+ + +	–		
12	187	+ +	+ +	+ + +	+ + +	+ + +	—		+ +
	189	—	—	—	—	—	—		+ +
	192	+ +	+	+	—	—	—		–
	193	+	—	—	—	—		+	+ +
	422	+ +	+ +	+ +	—	+ +			—
13	423	+ + +	+ + +	+ + +	+ +	+			—
	424	+	—	+ +	+	+ +			+
	441	+ + +	+	+ + +	+ + +	+ + +	+	—	—
	413	+ +	+ +	—	+ +	+ +	+	+ +	+ + +
14	414	+ +	+ +	+ +	—	+ +	–	—	—
	417	+ +	+	—	—	—	—	—	—
	418	+ + +	+ + +	+ + +	+ +	+ + +	–	—	—

Key for the semiquantitative evaluation

	Mononuclear cell infiltr	Glomerular changes	Vascular changes	Necrosis
+	small infiltrates	in less than 10 % of the glom	in a few vessels	patchy cortical necrosis
+ +	many or large perivascular	in 10–50 % of the glom	in many vessels	subcapsular zone
+ + +	diffuse infiltr	in more than 50% of the glom	in almost all vessels	partial necr of cortex/medul
+ + + +				total necrosis

cell infiltration glomerulitis or vasculitis was also present

Partial necrosis of the kidney with involvement of most of the cortex and outer medullary zone was seen in 15 allografts In 8 of these cases lymphocytic infiltrations glomerulitis or vasculitis was present In all cases the partial necrosis was accompanied by thrombosis of the intrarenal blood vessels

Total necrosis of the kidney was found in 10 allografts In 8 of these thrombosis of the intrarenal blood vessels was demonstrated, but no other sign of reaction could be discerned in the necrotic tissue In 2 allografts (nos 121 and 120 Table 1) with total necrosis microthrombi were present in about 50 per cent of the glomeruli

The necrotic areas of the grafts were demarcated by a zone of polymorphonuclear leucocytes in the above mentioned cases

Tubular degeneration could be demonstrated to a varying extent in most of the allografts Oedema was found in 89 allografts and severe hyperaemia in 12 allografts

Ten biopsies from 4 allografts (nos 443 444 445 and 446) demonstrated a gradual increase in the cellular infiltration in the glomeruli that were affected by proliferative changes

As indicated in Table 1 alterations in the vascular system of the kidney allografts regarded as a whole usually started as proliferative changes in the glomerular capillaries and were followed by proliferative changes in the endothelium of arteries and veins and by inflammatory exudative processes in glomeruli and vessels which in some cases were associated with thrombosis As a rule changes in allografts removed during the first four days after transplantation were less extensive than the changes in allografts removed later on Simultaneous with an increase in the



Fig 5 Allograft no 65 removed 11 days after transplantation Proliferative and exudative glomerulitis Heavy interstitial infiltration with large lymphocytes PAS × 400



Fig 6 Allograft no 157 removed 7 days after transplantation Severe endarteritis and heavy perivascular infiltration with small and large lymphocytes PAS × 100

severity of the lesions larger lymphocytes became the dominant constituent of the cell infiltrates

DISCUSSION

In many respects the qualitative changes demonstrated in our rabbit kidney allografts were similar to the changes described as being part of the allograft reaction in dogs (*Simonsen et al* 1953 *Dempster* 1953 and *Almgard* 1968), in rats (*Lindquist et al* 1968 *Feldman & Lee* 1968 and *Mahabir et al* 1969), and in rabbits (*Klassen & Milgrom* 1969)

We have previously reported the histopathological changes in rabbit *autografts* (*Lund & Myhre Jensen* 1970) Alterations in the cells of the intrarenal vascular system in some autografts resembled the alterations demonstrated in allografts suggesting that some kidney cells react similarly to both non immunological and immunological insults In autografts cellular infiltration was found in 30 per cent of the grafts proliferative changes in glomeruli or vessels in 20 per cent exudative changes and/or glomerular microthrombosis in about 10 per cent of the autografts

As regards the rabbit autograft the small lymphocyte was the most frequent type of cell in the early cellular infiltrates of the *allografts* Lymphocytic infiltration however appeared earlier and the number of cells increased more rapidly in allografts than in autografts This finding corresponds to the observations by *Semb et al* (1968) that lymphocytes were trapped in greater number in allo kidneys than in auto kidneys after perfusion of the organ with serum containing lymphocytes The trapping of lym

phocytes took place in the intertubular capillaries and venules of the juxtamedullary cortical zone lymphocytes were occasionally seen traversing the venule wall supporting the observation by *Feldman* & *Lee* (1968) that the large postcapillary venule is the main route for lymphocyte extravasation

The dominant cell of the cellular infiltrates changed from small lymphocytes to larger lymphocytes four to five days after transplantation This change is similar to changes observed in lymph nodes adjacent to the kidney graft (*Porter* 1965) and to changes observed *in vitro* in cultures of lymphocytes exposed to antigens (*Marshall et al* 1969) A similar change in the composition of the cellular infiltrates was demonstrated in only 2 out of 52 autografts

Mahabir et al (1969) observed that when large lymphocytes dominated the cellular infiltrate acute rejection frequently resulted whereas cell infiltrates composed mainly of small lymphocytes were not permanently injurious to the graft and in some cases these infiltrates were reversible in the experimental model of weak histo incompatibility in rats In our study dominance of larger cells from the fourth or fifth day after transplantation was often seen simultaneously with an acceleration of the rate of proliferative vascular changes and exudative changes in vessels and glomeruli

Guttmann et al (1969) have quantitated the differences in proliferation of mononuclear cells within renal allografts from rats of different strains and demonstrated that the greater the genetic disparity the greater the degree of cellular proliferation It is possible that the degree of cellular infiltration may also be an indicator of variations in histocompatibility in rabbits since the degree of cellular infiltration was usually

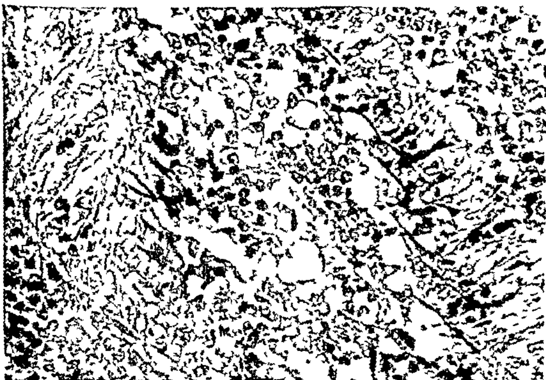


Fig [illegible] tail of Fig [illegible] Proliferated [illegible] ndothelial cells [illegible] ng bridge across th[illegible] lum[illegible]n Som[illegible] inflammatory [illegible] in th[illegible] ll of th[illegible] PAS [illegible] 400



Fig 6 Allograft no 157 removed 7 days after transplantation Severe endarteritis and heavy perivascular infiltration with small and large lymphocytes PAS × 100

severity of the lesions, larger lymphocytes became the dominant constituent of the cell infiltrates

DISCUSSION

In many respects the qualitative changes demonstrated in our rabbit kidney allografts were similar to the changes described as being part of the allograft reaction in dogs (*Simonsen et al* 1953 *Dempster* 1953 and *Almgard* 1968), in rats (*Lindquist et al* 1968, *Feldman & Lee* 1968 and *Mahabir et al* 1969) and in rabbits (*Klassen & Milgrom* 1969)

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across the lumina of the vessels In some cases the endarteritis resembled that of the early chronic allograft reaction (*Almgard* 1968)

Klassen & *Milgrom* (1969) also observed proliferative glomerulitis in the rabbit allo graft but apparently not as frequently as in this study perhaps because milder changes in the glomerular tuft were registered in our study

Exudative glomerulitis and vasculitis generally appeared later than proliferative changes The onset of exudative changes has been studied ultrastructurally by *Kount. et al* (1963) and *Porter* (1965) who found dissolution of the capillary endothelium followed by separation of the endothelial cells with exudation of fluid and cells to the interstitium of the kidney *Feldman* & *Lee* (1967) observed ultrastructural evidence of focal injury to thin walled vessels within the septa of rat allografts The lesions consisted of segmental swelling of endothelial cyto plasm and evulsion of the endothelium from its basement membrane which was frayed in these loci

The number of glomerular microthrombi in a rabbit allograft was generally small and in most cases associated with other glome rular signs of acute rejection In three cases however glomerular microthrombosis was observed in association with cortical necrosis and thrombosis of other intrarenal vessels but no other changes In two of these three cases microthrombi were seen in about 50 per cent of the glomeruli giving a picture resembling that of hyperacute rejection as originally described in the case of human allografts by *Kissmeyer Nielsen et al* (1966)

Seven of the allografts with thrombosis of the intrarenal vessels showed no necrosis which may indicate that thrombosis was recent Thrombosis of the intrarenal vessels without necrosis was not seen in the auto grafts

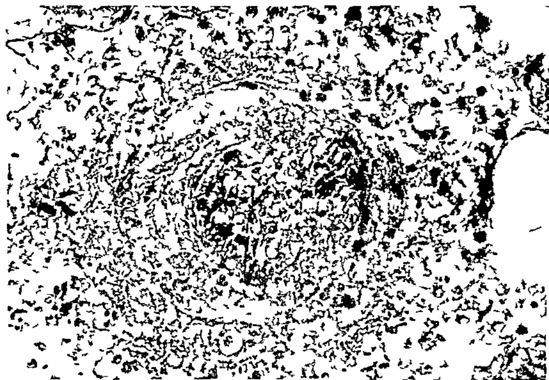


Fig 9 Allograft no 440 removed 4 days after transplantation Severe end rteritis and vasculitis with partial necrosis of the artery PAS × 400

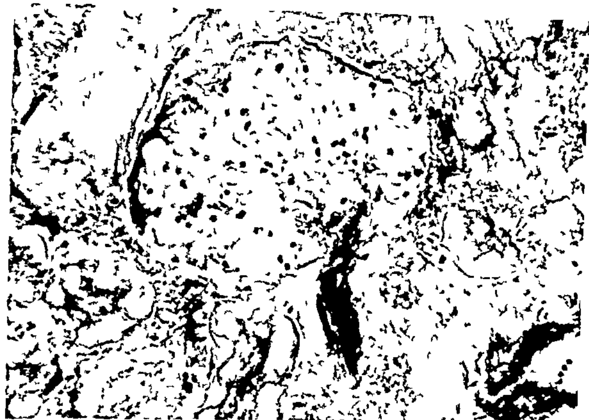


Fig 12 Allograft no 120 removed 8 days after transplantation Cortical necrosis thrombosis of the afferent arteriole and glomerular vascular pole Fibrinous exudate in Bowmann s space and in tubuli PTAH × 400

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THE EFFECT OF HEAT-LABILE SERUM FACTORS ON VITAMIN D-INDUCED ARTERIOSCLEROSIS IN MICE

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Administration of fresh guinea pig serum to vitamin D treated mice greatly promoted the induction of arteriosclerotic vascular changes Heat inactivated guinea pig serum had no such effect Since mice have minimal serum complement concentrations the results suggest a role of complement in vitamin D induced arteriosclerosis This hypothesis would be compatible with the earlier finding that decomplementation of rats prevents the development of vitamin D induced arteriosclerosis in these animals

Zymosan is known to inactivate circulating complement (*Pillemer et al* 1941) In a previous study (*Geertinger et al* 1970) it was shown that oral administration of massive doses of vitamin D to rats in a few days provoke histologically significant vascular changes particularly in the aorta and coronary vessels Such vascular changes were not observed in rats receiving intravenously administered zymosan in addition to vitamin D Thus it was suggested that complement is an important factor in vitamin D induced arteriosclerosis

Zymosan is however a biologically highly active substance and effects of zymosan other than that on the complement system must be taken into consideration In the present study we have therefore tried to approach the problem from a new angle Mice in contrast to rats and most other mammals possess a minimal haemolytic complement system (*Marcus et al* 1954 *Muschel et al* 1956) It is also an old observation that mice are peculiarly resistant to experimental arteriosclerosis compared to rats Mice would therefore be good experimental animals for studying the effect of complement in arteriosclerosis

The purpose of the present study was to investigate if intravenous administration of fresh complement to mice has any effect on the course of vitamin D induced vascular changes in these animals

Received 20 v 70

METHODS

Four week old male albino mice weighing about 25 gr were divided into 6 groups All the groups received standard food and water ad libitum The experiments lasted for 5 days Each group comprised 15 or more animals Vitamin D (Oleum calciferoli ultraconc 300 000 IU/ml) was dripped on small pieces of bread The animals consumed the bread within one or two hours if water and food had been withheld overnight As sources of complement were used fresh guinea pig serum or in some experiments hamster serum The mice had serum complement levels that were about 1/40 of those found in the guinea pig sera

Group 1 received about 800 000 IU of vitamin

D/kg bodyweight per os (about 20 000 units per animal)

Group II received vitamin D as group I and in addition intravenously administered complement (0 5 ml of guinea pig or hamster serum per day)

Group III received vitamin D and in addition intravenously administered inactivated complement (0 5 ml of guinea pig or hamster serum which had been heated at 56° C for 30 min)

Group IV received complement only (0 5 ml of guinea pig or hamster serum per day intravenously)

Group V received inactivated complement (heated guinea pig or hamster serum) only

Group VI Normal controls

At the end of the fifth day the animals were sacrificed with ether All the mice in groups I–III were clearly clinically sick with bristly fur and stiff legs The remaining mice (Groups IV–VI) were clinically unaffected

The heart and aorta were fixed in formalin and histological serial sections were performed on the aorta ascendens The sections were stained with von Kossa and van Gieson stains

RESULTS

Group I An increase in the distance between the elastic fibres of the media (heavy oedema) was noticed von Kossa staining showed compared with the normal controls a slight amount of calcium on the surface of the elastic threads but no calcareous deposits The smooth muscle cells between the elastic fibres were intact and no necroses or splitting up of the elastic fibres were observed (Fig 1) In one animal out of 16 one of the sections showed however rather severe changes of the kind described under group II

Group II In all the animals and in nearly all serial sections the elastic fibres had lost their ordinary concentric stratification The smooth muscle cells seemed to have disintegrated and especially the luminally situated lamina presented large necroses and splitting up (Fig 2) White blood cells and aggregates of thrombocytes were seen in abundance in many sections and were usually located along the intimal surface of the vessel wall Large calcareous precipitations were a prominent feature and were located particularly on the surface of the elastic fibres

Group III Histological changes similar to those in group I

Group IV–VI No significant histological changes (Fig 3)



Fig 1 Aorta ascendens of mouse after 5 days of large doses of vitamin D (group I) Heavy oedema but no necroses or calcareous deposits Smooth muscle cells intact (Cross section × 400 von Kossa)

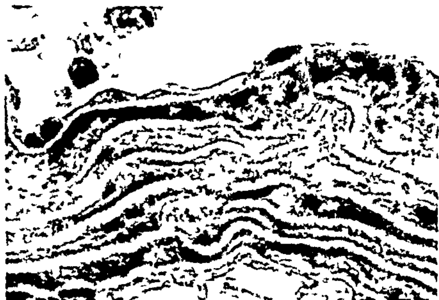


Fig ? Aorta ascendens after 5 da\s of ıntravenously admınıstered complement ın addıtıon to vıtamın D treatment (group II) \ote the total dısıntegratıon of smooth muscle cells splıttıng up of fibres and heavy calcareous deposıts partı cularly ın the lumınal (upper) part of the wall (Cross section × 400 \on Kossa)



F g 3 Aorta a cendens of normal control (Cros sectıon × 400 von Kos a)

DISCLSSION

xıc doses of vıtamın D pro\oke ın a few \s heavy \ascular changes ın the aorta of s Concurrent ıntra\enous admınıstratıon of zymosan pre\ents these changes thus ug gesting that complement ma} play a ke} role ın the appearence of \ıtamın D ınduced ar terıosclerosıs (*Geertinger* & *Sorensen* 1970)

BRIEF REPORTS

DOPAMINE IN A HUMAN ISLET CELL CARCINOMA METASTASIS*

Lennart Cegrell

By means of histochemical and chemical methods it has been demonstrated that the pancreatic endocrine cells in many species including man store biogenic mono amines particularly dopamine and 5 hydroxytryptamine (c f *Cegrell* 1968) The functional role of these substances in insulin and glucagon production is still unknown In an attempt to attack this problem a transplantable islet cell tumour of golden hamster was analysed and found to contain dopamine and 5 hydroxytryptamine as well as dopa and a mono amine like substance (*Cegrell et al 1969* a) which it has not been possible to characterize In addition to dopamine tyrosine hydroxylase and dopa decarboxylase *activities are present in the tumour indicating* that dopamine is synthesized by the tumour cells (*Cegrell et al* 1969 b *Axelsson et al* 1970)

The occurrence of 5 hydroxytryptamine in a human islet cell tumour producing insulin has been reported by *Gloor et al* (1964) and *Sluys Veer et al* (1964) found a patient with a malignant cell tumour producing hypoglycaemia and oid syndrome but dopamine has not been ated thus far in a human islet cell tumour An hepatic metastasis of malignant islet cell tumour (removed at autopsy from a patient treated with streptozotocin as reported by *Murray Lyon et al 1968 1970*) was placed in dry ice and sent to this laboratory where chemical determinations of the catechol derivatives were performed

Dopa and dopamine were determined according to *Anton & Sayre* (1964) and their identity was further established in a paper *chromatographic system described by Bertler et al* (1958) The concentration of dopa was 0 16 μg/g wet weigh of tissue and that of dopamine was 0 11 μg/g The paper chromatograms showed *two distinct spots* with the same characteristics as authentic dopa and dopamine *respectively In another determin*ation according to *Bertler et al* (1958) dopamine was found at a concentration of 0 22 μg g in an adjacent part of the metastasis

The occurrence of dopa and dopamine in the human islet cell carcinoma metastasis reported here together with the previous demonstration of 5 hydroxytryptamine in an i let cell tumou (*Glo r et al 1964*) *indicate that the human i let cell* carcinoma and the transplantable gold n hamster islet cell tumour are similar in the production of biogenic mono-amines

It is however difficult to compare the concentration of these substances in the two types of tumour since the human tissue was not obtained until 72 hours after death and the effect of this postmortem period is not known nor is the effect of streptozotocin treatment Thus it will be of great importance to analyse another tumour obtained either at operation or soon after death from a patient who had not received extensive drug treatment

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* Kindly sent to me by Dr *J Cassar* King s College Hospital London

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A POSSIBLE DISCRIMINANT TEST FOR HORMONE RESPONSIVE BREAST CANCERS ESTRADIOL—17 β SENSITIVE DEHYDROGENASE ACTIVITY

Sten Sander and Bjorn Skålhegg

A selection of breast cancer patients for hormonal treatment requires proper methods for discrimination between hormone responsive and hormone unresponsive tumours In search for clinically useful methods attention has been directed both towards the patient and the tumour itself The discriminant function test introduced by *Bulbrook et al* (1) is based on analysis of excreted urinary steroids

Measurement of estradiol uptake and binding in tumours conjugation of steroid hormones in tumours and tumour response to steroids in tissue cultures are other promising approaches currently under investigation (2 3 5 7)

A difference in estradiol metabolism was previously found between hormone responsive and – unresponsive breast tumours in rats (unpublished observation) In that study estrone was found in hormone responsive tumours after administration of estradiol whereas in hormone unresponsive tumours estrone could not be detected The enzyme estradiol 17 β dehydrogenase posesses the ability to transform estradiol to estrone and thus the activity of this enzyme might be of importance to or correlated to the hormone response of a particular tumour The present study describes a method for measurement of estradiol sensitive dehydrogenase activity (E SDA) in homogenates of breast tissue from rats with induced breast tumours This enzyme activity is correlated to the breast tumours response to oophorectomy

Material and methods

Breast tumours were induced in Sprague Dawley rats by intravenous injection of 7 12 dimethylbenzanthracene (DMBA) (5) When the tumours were 1 5 – 2 cm in diameter oophorectomy was carried out and the tumours were then classified as hormone responsive and hormone unresponsive as previously described (5) The animals were killed 1–2 weeks after oophorectomy and the breast tumours were rapidly taken out Wet weight was recorded The tissue was left in 4 ml of Krebs Ringer phosphate buffer pH 7 4 and treated in a Potter Elvehjem homogenizer and subsequently frozen Tissue for histological examination was fixed in 4 per cent formaldehyde embedded in paraffin and sections stained with haematoxylin and eosin

The frozen homogenates were thawed at 4 C and centrifuged at 20 000 x g for 30 min at 2 C in a Sorwall Refrigerated Centrifuge (model Rc 2) The supernatant was used as enzyme solution. The residue was checked for enzyme activity

Assay The enzyme activity was measured in a Hitachi Spectrophotometer (model 101) at 340 nm using a NADP/NADPH system at 37 C In order to avoid precipitation of the substrate 10 per cent of a mixture of propanediol/abs ethanol (1 1) was introduced in the assay mixture according to *Hagermann* (4) To get 100 per cent conversion of estradiol 17 β to estrone the keton binding agent hydrazine hydrate at a concentration of 0 1 M at pH 9 5 was used The complete assay system consisted of 2 5 ml of 0 1 M hydrazine hydrate 0 1 ml of NADP with final concentration 0 5 μmol 0 3 ml of propanediol/abs ethanol (1 1) containing 30 μg of estradiol 17 β

After 3 min of incubation 0 1 ml of tissue supernatant was added Readings were made at 1–2–3–4–5– and 10 minutes against the reference which was the complete system without the substrate The enzyme activity was determined from the slope of the initial linear part of the activity curve (difference in absorbance against time) One unit of enzyme activity was defined as the amount of the enzyme that will give a difference in optical density at 340 nm of 0 001/10 minutes at 37 C and pH of 9 5 This corresponds to about 0 5 mμmol of NADPH formed in the period During the incubation changes in the blank values were found which was probably due to the presence of other de

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E_2 SDA – Estradiol 17 β sensitive dehydrogenase activity

TABLE 1 *The Activity of E_2 SDA Is Given as Units ml⁻¹ 10 Min per 10 mg Tissue w/w Totally 55 Tumours Taken from 31 Animals Were Examined*

E_2 SDA	Hormone responsive tumours	Hormone unresponsible tumours	Response uncertain
0–14 Units ml 1/10 min	14	16	6
15–29 – –	4	0	0
30–59 –	7	0	0
60–220 – –	7	1	0

hydrogenases All activities given in table 1 is corrected for this blank value Each determination was performed in triplicate A possible activity of 3 β or 17 β hydroxysteroid dehydrogenases was also investigated using testosterone and epiandrosterone as substrates in a concentration 5 ug/ml assay mixture

Results

A total of 50 rats treated with DMBA yielded 55 adenocarcinomas in 31 rats In 17 growing tumours there were no distinct signs of regression 2 weeks after oophorectomy In 32 tumours histological atrophy with decrease in the average tumour diameter was evident 1–2 weeks after castration In six tumours the response to castration could not be properly assessed The diameter of these six tumours was not significantly influenced by castration and there were no distinct histological signs of regression

The dehydrogenase activity in the presence of 17 β in homogenized tissue in correlation to the oophorectomy response of the tumours is shown in Table 1 It will be seen that among the hormone responsive tumours 14 tumours have low enzyme activity whereas 18 tumours display a fairly high enzyme activity On the other hand only 1 out of 17 hormone unresponsive tumours showed high activity In the group uncertain response all six tumours had low enzyme activity

It is particularly interesting that in different tumours taken from a single rat high and low enzyme values were found to correlate well with the tumours response to castration In general the tumours with high levels of E SDA showed the most convincing response to oophorectomy Thus high enzyme activity in a tumour suggests that it belongs to the hormone responsive group The opposite conclusion however is not justified as low enzyme values were found in both tumour categories

Histologically the tumours with high levels of E_2 SDA could not be distinguished from the others

Some of the homogenates showed activity of 3 β and/or 17 β hydroxysteroid dehydrogenase but in general these activities were neither associated with the presence of E SDA nor correlated to the ovariectomy response

Normal breast tissue has been examined for E_2 SDA The values found were in the range 0–14 units which corresponds to the low values found in the majority of the examined tumours

Conclusion

It has been found that most but not all hormone responsive tumours can be selected from a tumour pool by our E SDA test Heterogeneity of the tumours may possibly explain the lack of conclusive results in some cases The method is fairly simple and can be used in routine laboratories It works with frozen tissue homogenates which makes transport to distant laboratories possible Besides clinical interest the observation of dehydrogenase activity in hormone responsive tumour tissue can be of general interest in cancer biology (3 7) We feel that the findings in this report should lead to further investigations and be extended to human breast cancer

We wish to thank cand mag *I Batnen* and *Jorunn Sander* for skilled technical assistance

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